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Caspase 3: potencial marcador para o sucesso da fertilização *in vitro*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Margarida Sâncio da Cruz Fardilha, Professora auxiliar convidada da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro e co-orientação da Dra. Helena Maria Sá Figueiredo, Responsável do Laboratório da Unidade de Medicina da Reprodução Dra. Ingeborg Chaves – Centro Hospitalar de Vila Nova de Gaia/ Espinho, EPE

Dedico este trabalho aos meus pais

o júri

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agradecimentos

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palavras-chave Apoptose, caspase-3, células do cumulus, fertilização *in vitro*, gravidez

resumo

Mundialmente é estimado que aproximadamente 70 milhões de casais tenham problemas de infertilidade, o que corresponde a um em cada sete casais em idade reprodutiva. O rápido aumento de problemas reprodutivos nas últimas décadas sugere uma maior probabilidade deste aumento ser devido a factores do estilo de vida e/ou ambientais, do que resultado de uma variação genética. Em Portugal 2,2% dos bébes nascidos resultam de técnicas de reprodução medicamente assistida. A transferência de vários embriões realizada, por vezes, no decurso destas técnicas pode resultar em gravidezes múltiplas, o que advém em complicações bem conhecidas para as mães e os bébes. São necessárias novas ferramentas de diagnóstico para que se possa transferir menos embriões com resultados similares ou melhores.

Neste estudo foi investigado o impacto de vários factores do estilo de vida no potencial reprodutivo de 47 casais que recorreram a técnicas de reprodução medicamente assistida.

Para além disso, foi realizada também, a correlação entre os níveis de expressão de um marcador da apoptose (caspase-3 clivada) nas células do cumulus do ovócito e a obtenção de uma gravidez (n=30). Uma concentração significativamente (p<0.01)) maior de caspase-3 clivada foi observada nas células do cumulus dos casais que não obtiveram uma gravidez.

Dada a dificuldade de obter respostas reais nos questionários dos voluntários e a pequena dimensão da amostra para avaliar parâmetros com tanta variação inter-individual, o estudo não conseguiu obter resultados estatísticamente significativos na correlação do impacto de factores do estilo de vida no potencial reprodutivo.

O estudo permitiu concluir que o nível de caspase-3 clivada nas células do cumulus parece ser um bom marcador da qualidade ovocitária e um bom predictor do resultado de gravidez.

keywords Apoptosis, caspase-3, cumulus cells, *in vitro* fertilization, pregnancy

Abstract

Worldwide it is estimated that approximately 70 million couples suffer from infertility, which corresponds to one in each seven couples at fertility age. The rapid increase in reproductive problems in recent decades suggests that they are more likely to be caused by lifestyle and/or environmental factors than as a result of genetic variations. In Portugal 2.2% of the babies born result from an assisted reproduction technology (ART) technique. The multiple embryo transfer performed sometimes in ART techniques may result in multiple pregnancies, which have well known complications for mothers and babies. New diagnostic tools are needed to improve embryo selection, in order to transfer less embryos to achieve similar or better results.

In this study the impact of lifestyle factors on the reproductive potential of 47 couples who resort to ART was investigated. Also, the correlation between the expression levels of an apoptotic marker (cleaved caspase-3) in oocytes cumulus cells and the achievement of pregnancy was performed (n=30). Significant (p<0.01) higher concentration of cleaved caspase-3 was observed in cumulus cells of couples who did not achieve pregnancy.

Given the difficulty in obtain reliable answers from the volunteers in the questionnaires and the small sample size to evaluate parameters with such a wide inter-subject variability it failed to give conclusive statistical significant data in the lifestyle impact into reproductive potential.

The present study allowed concluding that the level of cleaved caspase 3 in cumulus cells appears to be a good marker of oocytes quality and predictor of pregnancy outcome.

ABBREVIATIONS

AMH	Anti-Mullerian hormone
ART	Assisted reproduction technology
Acryl	Acrylamide
APS	Ammonium persulfate
Apaf-1	Apoptotic protease activating factor-1
ATP	Adenosine-5'-triphosphate
BCA	Bicinchoninic acid assay
Bisacryl	Bisacrylamide
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CatSper channel	Cation chanels of sperm
CC	Cumulus cells
Cdc2	Cyclin-dependent kinase 1
CHVNG	Centro Hospitalar de Vila Nova de Gaia
CNPD	Conselho Nacional de Proteção de Dados
CNPMA	Conselho Nacional de Procriação Medicamente Assistida
CO_2	Carbon dioxide
CRN1	Cannabinoid receptor subtype 1
CRN2	Cannabinoid receptor subtype 2
DAG	Diacylglycerol
dATP	2'-Deoxyadenosine-triphosphate

DNA	Deoxyribonucleic acid
DISC	Death inducing signalling pathway complex
ECL	Enhanced chemiluminescence
EDC	Endocrine disruptor
ESHRE	European Society of Human Reproduction
FADD	Fas- associated death domain
FF	Follicular fluid
FGF9	Fibroblast growth factor 9
GnRH	Gonadotropin-releasing hormone
hCG	Human chorionic gonadotropin
ICM	Inner cell mass
ICFBP	Insulin-like growth factor-binding protein
ICSI	Intracytoplasmic sperm injection
IMSI	Intracytoplasmic morphologically selected sperm injection
IVF	*In vitro* fertilization
IP_3	Inositol triphosphate
IU	International units
LGB	Lower gel guffer
LB	Loading guffer
LH	Luteinizing hormone
MII	Metaphase II
MPF	Maturation promoting factor
mRNA	Messenger ribonucleic acid

mΔ	Miliampere
Na^+	Sodium
N2	Nitrogen
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCOS	Polycystic ovary syndrome
PGCs	Primordial germ cells
PIP_2	Phosphotidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PLCζ	Phospholipase C zeta
RSPO1	R-spondin 1
SARP2	Secreted apoptosis-related protein-2
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SRY	Sex-determing region Y
SOX9	Sex-determining region Y- box 9
TEMED	Tetramethylethylenediamine
TBS	Tris-buffered saline
TBST	Tris-buffered saline with tween
THC	Δ9-tetrahydrocannabinol
UGB	Upper gel buffer
Wnt4	Wingless-type MMTV integration site family, member 4
WHO	World health organization

ZP	Zona pellucida
βhCG	Beta unit of human chorionic gonadotrophin

1. INTRODUCTION

1.1. HUMAN REPRODUCTION

Reproduction is the natural process by which new individuals are generated ensuring species perpetuation. In humans this is only possible by the fusion of two haploid cells: an oocyte and a spermatozoon. These cells have a complex process of formation only viable in a really specific hormonal and molecular atmosphere. This type of reproduction is denominated sexual reproduction and increases the genetic variability of species ensuring their survival through evolution.

1.1.1. SEXUAL DIFFERENTIATION

Biological differences between men and women are genetically determined during the embryonic development. In humans, embryos of both sexes follow the same evolution line until the sixth weeks, when the bi-potential gonads start to differentiate into testicle or ovary (Eggers *et al.*, 2012).

A high level of *Wnt4* (Wingless-type *MMTV* integration site family, member 4) and *RSPO1* (R-spondin 1) expression stabilizes cytoplasmic *β-catenin* which is translocated to the nucleo activating target genes expression. WNt4 and *β-catenin* suppress *SOX9* (sex determining region Y-box 9) and *FGF* (Fibroblast growth factor 9) *9* expression leading to ovary development. Male hormones absence leads to Wolffian ducts regression and Mullerian ducts development in female reproductive tract (Nef *et al.*, 2009).

In males the differentiation is determined by the gene *SRY* (Sex-determining region Y) specific of the Y chromosome. *SRY* stimulates *SOX9* and *FGF9* expression, responsible for the male gonad differentiation. Then the testicles start secreting hormones as testosterone and anti-Mullerian hormone (AMH). Testosterone stimulates the Wolffian duct differentiation in male reproductive tract and AMH induces Mullerian ducts regression (Schlessinger *et al.*, 2010) (Figure 1).

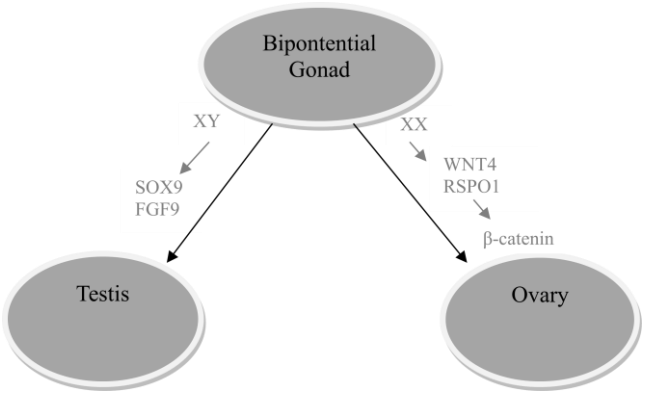

Figure 1| Schematic model of genetic sex determination.

1.1.2. FEMALE GONADAL DEVELOPMENT AND GAMETES PRODUCTION

The ovary development is characterized by massive colonization of the undifferentiated gonad with mesonephric cells (precursors of follicle cells), primordial germ cells migration into the genital ridge and gonadal sex differentiation (Palma *et al.*, 2012). After female reproductive tract is differentiated, between de 11-12 gestation weeks, the female gametes (oocytes) start to enter meiosis, that stops at the diplotene stage of prophase I. In this stage oocytes have flattened pregranulosa cells surrounding and this complex is called primordial follicle. They stay in this quiescent state until puberty, when the pituitary gland is stimulated to produce gonadotrophins after hypothalamus stimulation by gonadotrophin releasing hormone (GnRH). The gonadotrophins, follicle stimulation hormone (FSH) and luteinizing hormone (LH) together stimulate oocytes to restart meiosis. When the oocytes are at metaphase II the ovulation occurs (Hogarth *et al.*, 2010).

During early stages of folliculogenesis the follicle passes through three pre-antral phases: primary, secondary and tertiary follicles. These different stages have different morphological and structural characteristics. Primary follicle is characterized by the oocyte surrounded by a single layer of cuboidal granulose cells. When the primordial follicle is recruited, the granulose cells start to divide for mitosis and various genes are activated leading to a progressive increase in oocyte RNA synthesis. In this stage, genes encoding the zona pellucida proteins (ZP) are transcribed and translated. ZP proteins secretion polymerize near the oocyte ending to encapsulate it (Wassarman *et al.*, 1996). The secondary follicle consists in the oocyte surrounded by multiples layers of cuboidal or low columnar cells that form a stratified epithelium. At this stage, the follicle forms a theca layer (layer of stroma-like cells around the basal laminal of follicle). Theca cells produce androgens in response to LH, which neighboring granulose cells convert into estrogen by FSH-induced aromatase, being vital for the continuity of follicle development process (Young *et al.*, 2010). The follicle is designed tertiary once a cavity starts to appear in the middle of granulosa cells. This event marks the end of preantral stage (Kidder *et al.*, 2010).

Follicular antrum follows the oocyte enlargement due to division of follicular cells and the increase of follicular fluid (antral stage). A fully grown antral follicle, with a meiotic competent oocyte ready for ovulation, is designed pre-ovulatory or Graafian follicle (Rodgers *et al.*, 2010). The follicular fluid (FF) is an enriched microenvironment with nutritional and regulatory molecules, as well as apoptotic factors. The oocyte and the granulosa cells reside in the FF (Figure 2) and all the molecules must pass on their way to and from this microenvironment. The presence of fluid is crucial for the establishment of a separation between granulosa cells and mural granulosa cells, which limit the follicle wall and cumulus cells (CC) surrounding the oocyte. Antral stage is dependent on hormonal control by gonadotrophins as FSH and LH. FSH binds to its receptor on

granulosa cells, activating cAMP (Cyclic adenosine monophosphate)/ PKA (protein kinase A) pathway. This promotes cell proliferation, follicle cell and mural granulosa cells differentiation and acquisition of meiotic competence (Zuccotti *et al.*, 2011). The LH receptors are expressed on granulosa and theca cells. LH peak induces luteinization, cumulus cell-oocyte complex expansion, oocyte maturation and follicle rupture (Veeck, 1999; Noma *et al.*, 2011).

Usually in each cycle only one oocyte will complete meiosis I and arrest at metaphase II, when is ovulated. The ovulation is due to FSH and LH surge that promotes detachment of the cumulus oocyte complex from the follicular wall (Bras *et al.*, 1996; Fragouli *et al.*, 2012).

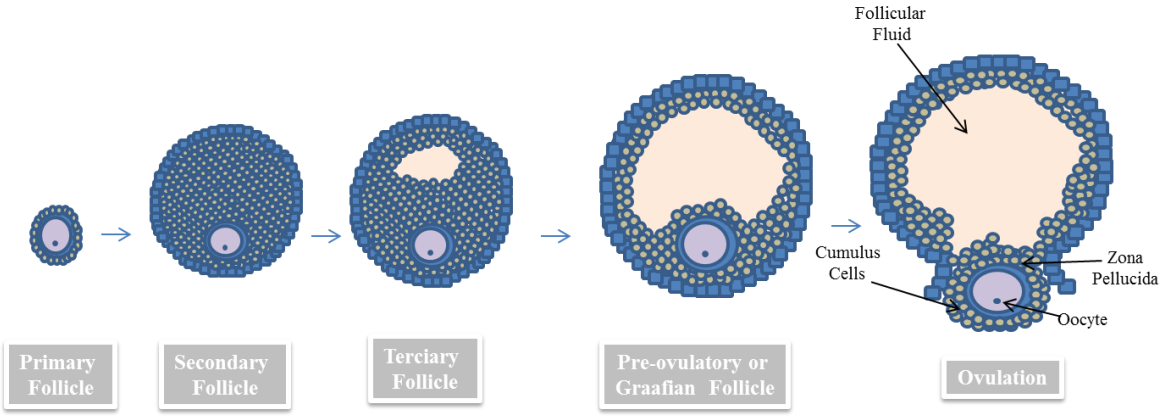

Figure 2- Folliculogenesis and ovulation.

The granulosa cells provide adequate physical and chemical conditions for oocyte development, changing their shape within the cycle phase. Granulosa cells vary from squamous type at primordial follicle to cuboidal type during ovulation and hypertrophied in the luteal stage. This cyto-differentiation and cells proliferation are essential to provide the support that oocyte needs during its growth and evolution from primordial to pre-antral stage (Huang *et al.*, 2010). The CCs have specific morphological and physiological characteristics and bidirectional exchange occurs between these two cells (Palma *et al.*, 2012).

In the follicle communication occurs through gap junctions, which consist in channels composed by proteins named connexins. The gap junctions directly connect the adjacent cells allowing diffusion of ions, metabolites and signaling molecules. These exchanges of regulatory and nutrient molecules are essential to oocyte growth and meiosis resume (Kidder *et al.*, 2010).

1.1.3. MALE GONADAL DEVELOPMENT AND GAMETES PRODUCTION

Testes differentiation happens between fifth to eighth gestation weeks when the progenitors of primordial germ cells (PGCs), derived from the epiblast of blastocyst that stays at mesonephros, migrate to the testis. Few steroidogenic Leydig cell precursors and epithelial somatic cells also migrate from mesonephros to gonad. This event marks the start of testicular vascular system organization. The somatic cells will differentiate into Sertoli cells that enclose the PGCs, forming seminiferous cords. Some of the interstitial somatic cells will differentiate into peritubular myoid cells, fibroblasts, endothelial cells or Leydig cells (Carmona *et al*., 2009).

After vascularization is complete, PGCs start to proliferate and after a few days they are arrested at G0/G1 cell cycle phase. These pre-spermatogonia cells, shortly after birth, resume proliferation and move to the basal lamina of the seminiferous tubules where they differentiate into spermatogonial stem cells. This pool of spermatogonial stem cells will only start spermatogenesis (spermatozoa production) 10 to 13 years after birth (Kolasa *et al*., 2012).

Once the puberty starts spermatogonias divide continuously by mitosis, increasing in number and improving the supply for new cells. The spermatogenesis begins when some of these cells stop dividing and differentiate into primary spermatocytes. Then these cells suffer a meiotic reduction originating two secondary spermatocytes. These cells will divide by meiosis producing four haploid spermatids (Bras *et al*., 1996). The spermatids need to undergo a structural differentiation to become mature spermatozoa (Figure 3). These morphological changes are called spermiogenesis and include spermatids elongation, flagella developing and cytoplasm residues elimination. All this processes happen at seminiferous tubules with Sertoli cells support. When the spermatozoa are mature, they are released into the tubule lumen. From here they migrates to the epididymis where undergo further maturation (O'Donnell *et al*., 2011). Spermatozoa are non-functional gametes by the time they leave the testis, and acquire progressive motility and ability to fertilize an oocyte only in the passage through epididymis the spermatozoa (Cornwall, 2009). These changes are due to alterations and redistribution of plasma membrane components and strengthened by extensive cross-linking of nuclear protamines and cytoskeletal structures in the tail. Also in this passage through epididymis sperm cell acquires signaling pathways necessary for spermatozoa being able to undergo capacitation (Naaby-Hansen *et al*., 2012). In humans the progression from spermatogonia to spermatozoon, takes approximately 74 days (Heller *et al*., 1964)

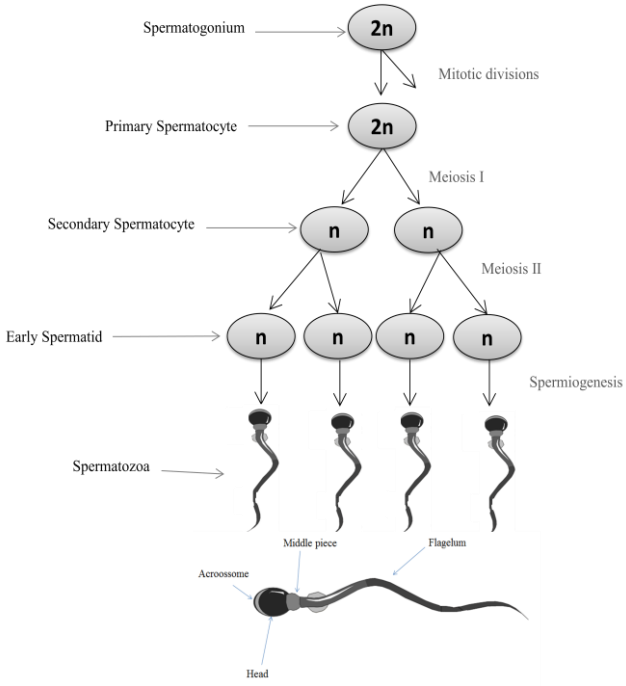

Figure 3- Spermatogenesis and spermiogenesis.

In males, LH triggers testicular Leydig cells to produce and secrete testosterone. The action of testosterone and FSH on their receptors of Sertoli cells leads to spermatogenesis progression (McLachlan *et al*., 2002). Testosterone is also released into circulation and acts as a negative feedback on the pituitary gland suppressing further LH secretion (Jequier *et al.*, 1986).

Men ejaculate is formed by spermatozoa and seminal plasma, which is formed by 1/3 of prostatic secretion and 2/3 of seminal vesicles secretion. Prostatic secretion has low pH and contains citric acid, acid phosphatases and zinc. Seminal vesicles secretion is viscous, has high pH and contains fructose, which is essential for spermatozoon progression (Bras *et al*., 1996).

1.1.4. SPERMATOZOA CAPACITATION

Spermatozoa undergo the maturation after achieving the female genital tract. This process is responsible for providing spermatozoa: hiperactivated motility (necessary to reach the Fallopian tubes); the ability of be guided by thermotaxis and chemotaxis; the capacity of penetrate cumulus layers surrounding oocyte; and the power to undergo acrossomal reaction and bind the oocyte. The hyperactivated motility is probably due to an elevation in intracellular calcium (Ca^{2+}) through CatSper Ca^{2+} channel (cation channels of sperm) and by the intracellular Ca^{2+} stored at the nuclear envelope (Armon *et al*., 2011).

1.1.5. FERTILIZATION

Fertilization is the fusion between an oocyte and a spermatozoon, resulting in a fusion of their nuclear material. The oocyte ovulated is surrounded by CC, which are enclosed in a matrix of polymerized hyaluronic acid and remains in the oviduct. This mucopolysaccharide matrix can be broken by the enzymatic action of sperm acrossome hyaluronidase. Due to hyaluronidase action and the hyperactivated motility, spermatozoa are able to pass through this mass of cells (Liu, 2011). When they achieve ZP, the spermatozoa need to recognized glycoproteins. In humans the ZP is composed by four glycoproteins: ZP1, ZP2, ZP3 and ZP4. The recognition of sperm cell and induction of acrossome reaction happens when sperm binds to ZP1, ZP3 and ZP4. The acrossome reacted spermatozoon is able to bind to ZP2 that acts as a secondary sperm receptor (Gupta *et al*., 2012). After reaching the periviteline space spermatozoa ends fusing with oolema by a fertilin/integrin that mediated adhesion process (Liu, 2011).

Once a spermatozoon fuses with an oocyte, a process of blockage to the binding of other sperm cells is initiated, preventing polyspermy. Many studies tried to explain the mechanisms of this blockage. In many species two kinds of mechanisms where identified: a "fast polyspermy block" and a "slow polyspermy block". The first one is characterized by a depolarization of oocyte membrane due to sperm bind, resulting in a Na^+ (sodium) influx, which changes the membrane potential from negative to positive. This event prevents that more sperm attaches the oocyte membrane. However is not clear that this mechanism occurs in mammals (Mio *et al*., 2012). The "slow polyspermy block" involves an oscillation of Ca^{2+} levels due to sperm binding. This triggers the activation of cell cycle, through degradation of the catalytic subunit cyclin-dependent kinase 1 (Cdc2) of Maturation Promoting Factor (MPF) and the exocytose of cortical granules (localized under the oolema) to the periviteline space. These cortical granules contain enzymes as hydrolase, proteinase and peroxidase. The enzymes act modifying sperm receptor structure (ZP2, ZP3) removing sperm binding capacity and leading to a hardening of ZP. These mechanisms change the receptivity of the oocyte and form a hardened protective layer that confers protection to the developing embryo (Dale *et al*., 2011). The oscillations of Ca^{2+} levels are believed to be caused by the hydrolysis of phosphotidylinositol 4,5-bisphosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG), leading to the IP_3-mediated Ca^{2+} release from intracellular Ca^{2+} stores, such as the endoplasmic. The factor that causes this phenomenon is believed to be a soluble factor sperm specific, the isozyme phospholipase C zeta (PLCζ) (Ramadan *et al*., 2012).

1.1.6. FROM ZYGOTE TO PREGNANCY

The spermatozoon entrances into the MII oocyte (Figure 4(a)), activates it, and induces the completing of meiosis II, with the 23 double-stranded chromosomes being split on their centromeres and the chromatids are separated (half to the oocyte and half to the second polar body). Male and female pronuclei are formed (the male pronucleus forms near the site of sperm entry and the female forms at the ooplasmic pole of the meiotic spindle). Both pronuclei migrate to the center of cell (Figure 4(b)), entry in close contact and lose their membranes, as they enter in syngamy. At this stage the maternal and paternal chromosomes reorganize and pair (Veeck, 1991). The oocyte stored maternal messenger Ribonucleic Acid (mRNA) and energy precursors. This cell has repair mechanisms that turn possible the modification of epigenetic marks on the paternal genome (McGinnis *et al.*, 2011).

Figure 4- Embryo evolution: (a)normal MII occyte; (b) normal fertilized oocyte with two pro-nuclei; (c) 4 cells embryo; (d) 8 cells embryo; (e) morulae; (f) blastocyst.

The embryo starts to cleave around 25-27 hours after insemination and the following cycles take about 18 hours. Ideally, attending to the insemination time, an embryo should have 4 cells at 44 hours (Figure 4(c))and 8 cells at 68 hours (Figure (d)) (ALPHA, 2011). Studies in many mammals' species indicate that the first cleavage is controlled by the human sperm centrosome. In the first three cleavages the embryo passes through a series of mitotic divisions with a reducing of approximately 28.5% of blastomeres volume, resulting maintenance of overall size. During these firsts cleavage divisions the blastomeres are totipotent cells; this potency is lost when cells start to interact with one another in the compaction process (Veeck, 1999). The embryo development depends on initiation and regulation of new embryonic genome transcription. The zygote genome activation occurs progressively with the depletion of maternal Ribonucleic Acid (RNA) transcripts and the transcription of new embryonic mRNA, with the shift in protein synthesis and post-translational modification, and with the development of a functional nucleossome structure (nuclear organization region). In humans, is believed that the timing of genome activation starts at 4-cells stage (Elder *et al.*, 2003).

In the Day-4 the blastomeres contact to each other's intensify with a consecutive reduction of the intercellular spaces and a blurring of the cell contours. The compactation process is Ca^{2+} dependent

and requires involvement of the cytoskeleton and the adhesion molecule cadherin. In this stage the embryo is designed morulae (Figure 4(e)). While cell division continues, epithelioid properties are acquires by the outermost layer of cells, the trophectoderm. This layer has tight junctions and desmosomes making impossible the contact between the outside environment and the inner space. A cavity of liquid, the blastocele, is formed by the actively pumping of salts and water. The innermost cells form an eccentrically placed cluster, the inner mass cell (ICM). At this stage the embryo is designed the blastocyst (Figure 4(f)), and normally happens at Day-5. The ICM originates the embryo and the chorion, the trophectoderm originates the placenta and is responsible for the embryo implantation in uterus. While embryo keeps dividing, it migrates through the ciliated epithelium of the oviduct until reaching the uterus 4 to 5 days after fertilization (Bras *et al*., 1996).

The blastocyst increases in overall size at later stages, due to accumulation of fluid. During embryo evolution the ZP becomes a thin outline, which seems to be due to physical pressures exerted by blastocyst expansion and the effect of lytic enzymes produced by the embryo (Veeck, 2003; Sireesha *et al.*, 2008). Meanwhile the blastocyst begins to expand and contract forming a blastocoelic tension. The rupture and escape of blastocyst from the ZP is named hatching. Being free of the ZP the embryo is ready to implant in the uterine wall (Veeck, 2003).

Implantation is the result of complex interactions between blastocyst and uterine cells. The embryo development needs to be synchronized with endometrium state. The endometrium receptivity depends of hormonal stimulation and communication with blastocyst by several factors as cytokines, growth factors and adhesion molecules. In a first step the blastocyst appears to orientate itself (orientation) and apposes to the endometrial surface (apposition). Then the embryo starts to firm adhesion contacts and translated into firm adhesion sites (adhesion). Finally, the embryonic trophoblast penetrates the ephithelium and the basement membrane, leading the embryo to invade the underliyng stromal cells (invasion). The trophoblast differentiation allows the formation of a connection with maternal vasculature and placenta formation (Grewal *et al.*, 2008).

(In)Fertility European Society of Human Reproduction and embryology (ESHRE) defines infertility as "A disease of the reproductive system defined by the failure to conceive after 12 months of regular unprotected sexual intercourse." (ESHRE, 2013).

It is estimated that worldwide approximately 70 million couples suffer from infertility, which correspond of one in each seven couples at ferlility age. Infertility represents not only a physiological and psychological but also a serious problem in the population growing proportion.

To deal with this social and economic problem governments around the world are investing heavily in assisted reproductive technology (ART), which helps couples to deal with this problem, and providing scientists verbs to study and better understanding the human reproduction, trying to find ways to diagnose and treat infertility causes (Kashir *et al.*, 2010). In the last ESHRE data from 2008, 1.6% of European births are related to ART, children who correspond to 64526 infants (Ferraretti *et al.*, 2012). In the report from Conselho Nacional de Procriação Medicamente Assistida (CNPMA) related to the data from 2010 the babies from ART techniques correspond to 2.2% of total births registered in Portugal in that year (CNPMA, 2012).

Infertility can be due to various etiologies (Figure 5).

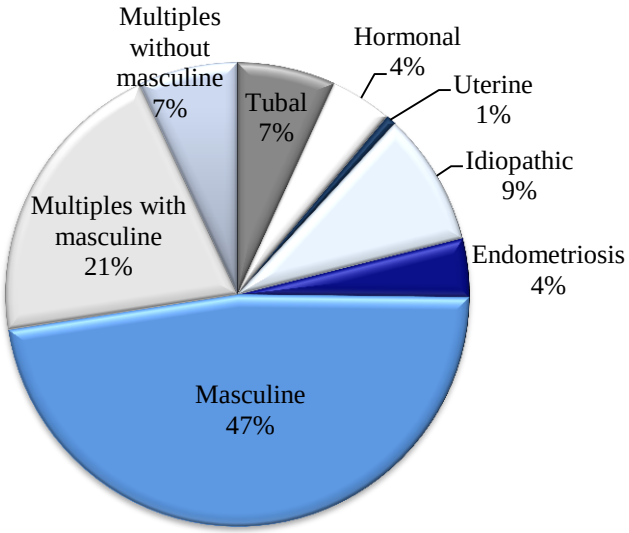

Figure 5- Causes of infertility at Centro hospitalar de Vila Nova de Gaia (CHVNG) in the last ten years (2002-2012).

1.1.7. FEMALE INFERTILITY

Tubal pathology is a common cause of female infertility. This pathology is characterized by an anatomic abnormality that makes impossible the union of the sperm with the oocyte. A previous history of pelvic inflammatory disease, *Chlamydia spp.* infection, abortion, ectopic pregnancy or tubal surgery (Yi *et al.*, 2012) is common in this etiology.

Hormonal dysfunctions lead to ovulatory failures. The hormonal pathology can be divided in three principal categories: hypogonadotropic hypogonadism characterized by reduced activity of the hypothalamus and pituitary resulting in ovary under stimulation; hypergonadotropic hypogonadism

that consists in ovarian failure due to lack of ovarian response, which results in loss of the negative feedback and a consecutive rise of gonadotrophin levels; and normogonadotropic hypogonadism name given to the abnormal ovarian activity, the main epidemiology in this group is the polycystic ovary syndrome (PCOS) (Palihawadana *et al.*, 2012). This pathology is the most usual endocrinopathology, affecting up to 10% of women on their reproductive age (Carmina, 2012). PCOS is characterized by oligo-anovulation, hyperandrogenism and the presence of polycystic ovaries. PCOS patients may be subfertile due to metabolic, inflammatory and endocrine abnormalities action on ovarian function and their impact in oocyte quality and endometrial receptivity. Pregnancy-associated risks appear to be also higher in these patients (Fauser *et al.*, 2012).

Uterine anomalies are associated with infertility, recurrent miscarriages and prematurity. These can be due to multiples pathologies, such us the presence of fibroids, intrauterine synechiae and endometrial polyps in the uterus (Brinsden, 1999). The uterus shape can also be altered, mainly due to the presence of septus or anomalous shape (septate, arcuate and bicornuate uterus), which are the result of Mullerian ducts malformations (Raga *et al.*, 1997).

Endometriosis is a gynecological pathology where endometrial glandular and stromal cells exist in the extra-uterine environment (most commonly in the ovaries and on the surface of pelvic cavity organs). Is estimated that 10-15% of females on reproductive age suffer from this disease. Endometriosis alters the hormonal axis and is responsible for the production of multiples apoptotic factors, which result in less oocytes ovulated, impair fertilization, poor embryo quality and implantation, increasing pre-term lost and miscarriage (Stilley *et al.*, 2012).

The oocyte quality has a direct impact on embryo evolution and implantation. Grading oocyte quality can be determinant on ART results (Zuccotti *et al.*, 2011). Classicaly, only morphological parameters are normally taking into account, because is a quick and simple evaluation, besides identifying more frequently negative predictors of oocyte quality than positive. There are many studies that try to identify specific molecular markers of oocyte quality, but the techniques are complicate, time consuming and require expensive laboratory equipment, reasons that turn this techniques inapplicable in the daily clinical practice. Novel non-invasive techniques of oocyte quality that provide results in real time are urgent to help identifying the gametes/embryos with best potential to achieve a pregnancy (Revelli *et al.*, 2009).

1.1.8. MALE INFERTILITY

The last manual for examination and processing of human semen of World Health Organization (WHO, 2010) defines a normal semen sample as described on Table 1. The main semen anomalies (Table 2) can reside at concentration (azoospermia and oligozoospermia), motility (asthenozoospermia) and at morphology (teratozoospermia) (WHO, 2010). Abnormal semen characteristics result from multiples pathologies, such as anatomical, hormonal and genetic causes.

Table 1| Reference values (WHO, 2010).

Volume	1.5ml
pH	≥7.2
Concentration	$\geq 15x10^6$/ml
Vitality	58
Progressive Motility	≥32%
Normal Morphology	≥4%

Table 2| Semen anomalies nomenclature (adapted from WHO, 2010).

Asthenozoospermia	Progressively motile spermatozoa percentage below the lower reference limit
Azoospermia	No spermatozoa in the ejaculate
Oligozoospermia	Total number (or concentration, depending on outcome reported) of spermatozoa below the lower reference limit
Teratozoospermia	Morphologically normal spermatozoa percentage below the lower reference limit

In the group of anatomic pathologies some dysfunctions can be identified, such as: retrograde ejaculation (situation where spermatozoa enter into the bladder instead of going to the urethra and be ejaculated (Edwards *et al.*, 1995)); epididymis obstruction or absence (pathologies which can be due to inflammatory infections, congenital vas deferens absence and cystic fibrosis (Casals *et al*., 2000)); cryptorchidism (refers to a situation where testis do not migrate into the scrotum and permanence in the abdomen where temperature is 2-3ºC higher, which can cause testicular dysfunction (Ferraz *et al*., 2010)); hypospadias (ventrally placed urinary opening due to a premature fetal arrest of the urethra development (Adamovic *et al.*, 2012)).

Among the hormonal pathologies the most common are hypogonadotropic hypogonadism (characterized by low circulating gonadotropins and testosterone) and hypergonadotropic hypogonadism (this condition of high levels of FSH can be due to primary gonadal failure or damage on Sertoli cells) (Casals *et al.*, 2000).

Finally, the genetic causes more often are 47, XYY karyotype (men can be fertile but usually have oligo or azoospermia); 47, XXY karyotype or the Klinefelter syndrome (greatly related with impaired spermatogenesis leading to oligo or azoospermia) and Y chromosome microdelections (normally a degenerative pathologie leading to azoospermia during life (Martin, 2008)).

The parameters commonly used in ART laboratories only give general information on the spermatozoa quality and do not provide any information about sperm deoxyribonucleic acid (DNA). Spermatozoa are a singular cell that undergoes a protamination process, in which the mechanisms of DNA repair are absent. During the transit of spermatozoa through epididymis and female reproductive tract all damages that occur are not repaired. The oocyte can repair some of these damages, but all depends on the extension and gravity of them. High levels of DNA fragmentation are evidently correlated with lower fertilization rate, poor embryo quality, impaired implantation and increased abortion rate (González-Marín *et al.*, 2012). Nowadays new technologies provide more information about cells and spermatozoa is not an exception, example of that is the ability to identify epigenetic alterations and their direct correlation with infertility. These epigenetic changes result in abnormal embryogenesis and can be due to environmental toxins and aging. Further studies that focus on the etiologies of infertility are urgent to improve the treatment of infertility pathologies (Jenkins *et al.*, 2012).

1.1.9. IDIOPATHIC INFERTILITY

It is estimated that in 15 to 30% of infertile couples specific medical causes for infertility are not identified with standard infertility evaluation. The treatment of this unexplained or idiopathic infertility is mainly empiric (Quaas *et al.*, 2008).

1.2. ART TECHNIQUES

In 1980 Edwards *et al.* published a study relating human pregnancies achieved after transfer of human embryos fertilized and cleaved *in vitro*. From this pregnancies resulted in United Kingdom the first baby born from ART techniques, Louise Brown, at 25 July 1978 (Steptoe *et al.*, 1978). In Portugal ART techniques started only on 1985 at Santa Maria Hospital, Lisbon. The first baby was

born in 1986 (CNPMA, 2013). *In vitro* fertilization (IVF) technique started a new era in infertility treatment, what was recognized on 2010 with the award of Nobel Prize for "Physiology or Medicine" (Gianaroli *et al.*, 2010), being attributed to Robert Geoffrey Edwards.

IVF technique shows some limitations when a serious male factor is present resulting in really poor results and sometimes the gametes even fail to interact completely. In 1992 Palermo *et al.* described pregnancies resulting from a technique of intracytoplasmic sperm injection (ICSI) characterized by the direct injection of a single spermatozoon into the ooplasm. This technique allows men with really few live sperm to achieve fertilization and parenthood, being the most efficient technique for male infertility factor (Mansour *et al.*, 1998).

1.3. STATE OF ART

Despite several ART techniques improvements over last years, live birth rate is still around 30-40% (Ferraretti *et al.*, 2012). This obliges couples to undergo several treatments to achieve a pregnancy, which increases their emotional stress. Financial costs cannot be forgotten, either supported by the couple or by National Health Service.

Currently, in almost laboratories around the world, the selection of embryos for transfer is only based in the rate of embryo cleavage and their morphology (Wang, 2011). In order to avoid multiple pregnancies associated with serious health complications for mothers and babies, there is a tendency in some centers to transfer a single embryo (Ajduk *et al.*, 2012).

Polities of single embryo transfer need all resources to choose the embryo most likely to result in a pregnancy. All technology available in investigation laboratories needs to adapt to ART laboratories giving results from really low quantities of sample available and in really time. Presently, in investigation laboratories we are living in omics era with: genomic, trancriptomic, proteomic and metabolomic tools (Appendix 1). These technologies are becoming, potentially, very useful diagnostics tools, investigating differences among follicles, oocytes and embryos by studying follicular fluid, cumulus cells and media of embryo culture. The differences shown by this analysis can be determinant for embryo selection (Seli *et al.*, 2010).

Regarding the spermatozoa, a technique of high-magnification sperm selection had emerged, intracytoplasmic morphologically selected sperm injection (IMSI). This technique enables the identification of abnormal proportions of sperm head size, mid-piece abnormalities and/or the presence of vacuoles in the sperm head (which are correlated with lower pregnancy rates). Some studies refer an increase in embryo quality, pregnancy and implantation rates with this technique

(Wilding *et al*., 2011). Controversially, other studies clearly defend that there is no difference in oocyte fertilization rate or embryo development between IMSI and ICSI (De Vos *et al.*, 2013).

1.4. APOPTOSIS

Apoptosis is a specific way of cell death essential for organism survival. In apoptosis the cell dye in an energy dependent way but the formation of apoptotic bodies encapsulating the cell content lead the cell death without an inflammation process that can kill neighbour cells. It is a vital process for normal cell turnover, proper development and immune system function, mutated cells control, hormone-dependent atrophy, normal embryonic development and maintenance of cell homeostasis (Rastogi *et al.*, 2009).

There are at least two broad pathways that lead to apoptosis: an intrinsic pathway and an extrinsic pathway (Figure 6). The intrinsic pathway is characterized by a mitochondrial dysfunction which leads to cytochrome c release from mithocondrial intermembrane space into cytosol. Cytochrome c free from mithocondria binds to the apoptotic protease activating factor-1 (Apaf-1). This event triggers the formation of the apoptossome, a 7-spansymmetrical active complex in nucleotide dATP/ATP dependent manner. The apoptossome recruits procaspase-9 into its central region to form a holoenzyme. The apoptosome-bound procaspase-9 is activated which ledas to activation of effector caspases (e.g - caspase 3) (Lawen, 2003). Caspase-3 is a critical executioner of apoptosis, as it is either partially or totally responsible for the proteolytic cleavage of many key proteins, such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP). The extrinsic pathway starts with the binding of toxic molecules to a death receptor on the cell surface. Activation of these cell death receptors promotes the formation of the death inducing signalling pathway complex (DISC), composed for Fas-associated death domain (FADD) and caspase-8. This caspase-8 binding leads to its activation turning caspase-8 able to activate directly the effector caspases or indirectly through the cleavage of BH3- only protein Bid (Niers *et al.*, 2011).

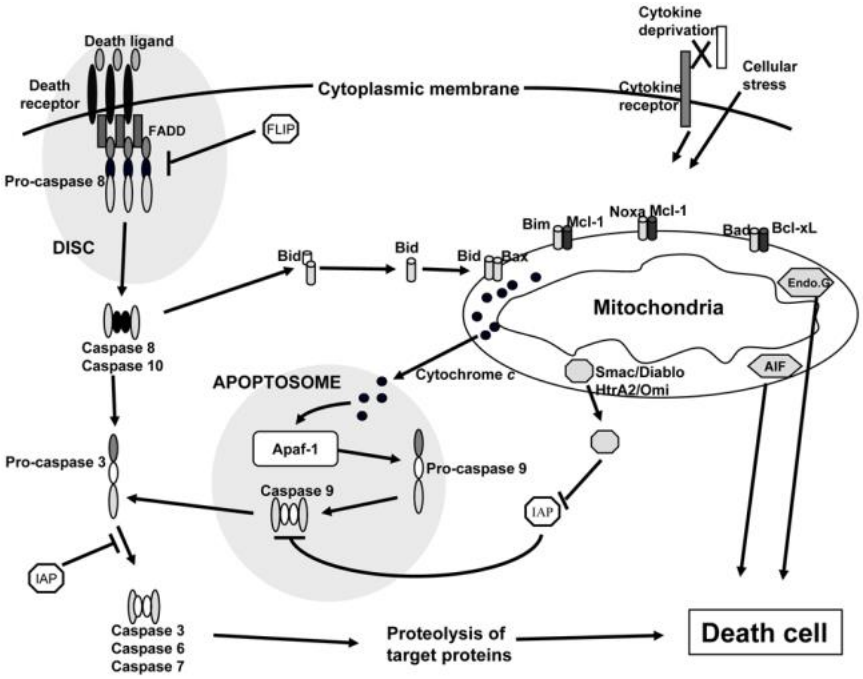

Figure 6- Apoptosis extrinsic and intrinsic pathway (Jourdan *et al*., 2009).

Both apoptotic signalling pathways converge in specific proteases, the caspases, that are cysteine proteases with the ability to recognize and cleave after aspartic acid and play a key role in the initiation and execution of apoptosis. Caspases can be functionally divided in two classes: the initiator caspases (long prodomains containing DED domains or a caspase recruitment domain (CARD)) and the executioner caspases (short prodomains) (Lawen, 2003).

In the females during fetal life, apoptosis occurs on oocytes, but during adult life it is mainly detected in granulosa cells of secondary and antral follicles (Hussein, 2005).

1.5. AIMS

The aim of this study was to investigate the impact of lifestyle factors on the reproductive potential of couples who resort to assisted reproductive techniques and to explore the correlation between the expression levels of cleaved caspase-3 in CC and achievement of pregnancy.

2. MATERIAL AND METHODS

2.1. STUDY OVERVIEW

Experimental procedures were performed in Signal Transduction Laboratory, Center for Cell Biology, University of Aveiro (Aveiro, Portugal) and in Unidade de Medicina da Reprodução Dra. Ingeborg Chaves, CHVNG (Vila Nova de Gaia, Portugal).

2.2. HUMAN SAMPLES COLLECTION

In the study 47 couples WHO underwent IVF/ICSI treatments at CHVNG were included. Investigations were performed with the approval of the institutional research ethics board of CHVNG, and the Conselho Nacional de Proteção de Dados (CNPD) (Appendix 2). All couples signed a written consent authorizing the use of the samples for research purposes (Appendix 3).

2.3. QUESTIONNAIRE

All patients answered a questionnaire by assisted interview (Appendix 4), however some answers may not be completely true.

2.4. STATISTICAL ANALYSIS

Data was first summarized with traditional descriptive methodology. Relationships between the main outcomes and predictor variables and environmental risk factors were explored with univariate methodology: Independent samples Student T-tests, Mann Whitney U tests, Kruskal-Wallis tests, ANOVA, chi-square and Fisher exact tests, depending on their distributions. The effects of the risk factors on the predictor variables were explored by employing the same hypothesis tests as well as correlation coefficients.

P-values less than 0.05 were considered to be statistically significant.

STATA/SE 12.0 for Windows by StataCorp LP, Texas, USA was used for all statistical analysis.

2.5. CLINICAL METHODOLOGY

2.5.1. FOLLICULAR ASPIRATION

In order to obtain more oocytes than in a natural cycle a hormonal stimulation was performed. Exogenous gonadotrophins induced multiple follicles stimulation and provided support for their development. When at least one follicle achieved the diameter of 17mm, human chorionic gonadotrophin (hCG) was administered and 34-36 hours after, a transvaginally follicular aspiration with ultrasoundguided was performed for oocyte retrieval.

2.5.2. OOCYTE ASPIRATION

Using a needle attached to a vaginal ultrasound, the ovary was reached and the FF present in the follicles was aspirated and immediately examined under a stereomicroscope of the workstation (Workstation L126, KSystem) in order to identify the oocytes. The oocytes were washed in G-MOPS^{TM} (Vitrolife, Sweden) and incubated in a multidisc (Nunc, Thermo Scientific) with G-IVF^{TM} (Vitrolife, Sweden) at 37ºC, with 5 % CO_2 (Heracell 150, Heraeus), for 2-3 hours.

2.5.3. SPERM PREPARATION

Semen samples were collected by masturbation into a sterile container. After liquefaction, the semen was first centrifuged (Labofuge 400, Heraeus) with gradients PureSperm®100 (Nidacon, Sweden) at 40% and 80%, for 20 minutes at 300g. The supernatant was carefully aspirated and the sperm pellet re-suspended and washed with Sperm Preparation Medium® (Origio, Denmark) for 10 minutes at 300g. The supernatant was again removed and Sperm Preparation Medium® (Origio, Denmark) was carefully added above the pellet and incubated at 37ºC in an angle of 45º (Swim-up technique). After 30-60 minutes the supernatant was aspirated and put in incubator at 37ºC, after calculation of spermatozoa motile concentration.

2.5.4. INSEMINATION

In the IVF technique a calculated volume of the final motile sperm preparation was added to the oocytes, in order to achieve $10x10^6$ motile spermatozoa/ml.

In the ICSI technique the first step was to evaluate the nuclear maturity of the oocytes. For that, oocytes were denuded enzimatically with SynVitro®Hyadase (Origio, Denmark) in order to

remove the CCs from the oocyte. Only oocytes that presented a clear first polar body (indicative of MII stage) were injected with sperm chosen and immobilized in PVP Medium (Origio, Denmark) under an inverted microscope (Diaphot 300, Nikon) and with the help of a hydraulic system (Narishige, Nikon).

2.5.5. FERTILIZATION AND EMBRYO

An oocyte was considered normally fertilized when two pronuclei and two polar bodies were visible after 17+1h of insemination. These zygotes were cultured in individual droplets of G1™ (Vitrolife, Sweden). The embryos were daily evaluated specifically at 44h after insemination on day 2 and at each 24h forward. According with the number and embryo quality the transfer was realized between day 2 and day 5. Embryo selection was prolonged if there were many embryos of high quality and the option of embryos cryopreservation was discussed with the couple. Attending to the couple history of infertility, female age and couple desire, one or two embryos were transferred to the uterus under ultrasound guidance.

2.5.6. PREGNANCY

After 12-14 days of oocytes collection a blood analyzes for beta unit of human chorionic gonadotrophin (βhCG) level took place. A positive result in the βhCG was followed by an ultrasound ecography at day 30. The women were only considered pregnant when at least one gestational sac was visible.

2.6. MOLECULAR METHODOLOGY

2.6.1. CUMULUS CELLS COLLECTION AND STORAGE

The CCs were obtained from the FF under a stereo microscope in a workstation (Workstation L126, Ksystem). All free CCs were collected, as well as some CCs attached to the oocytes that were separated using a mechanical denudation with needles. The cumulus cells were washed twice with PBS (Sigma–Aldrich, Portugal) at 300g for 6 minutes (Labofuge 400, Heraeus). Finally the washed pellet of CCs was separated into two aliquots: 10 µl was used directly on the coverslips for the immunocytochemistry assays and the reminiscent pellet was frozen and stores at -196ºC, in N_2 (Figure 7).

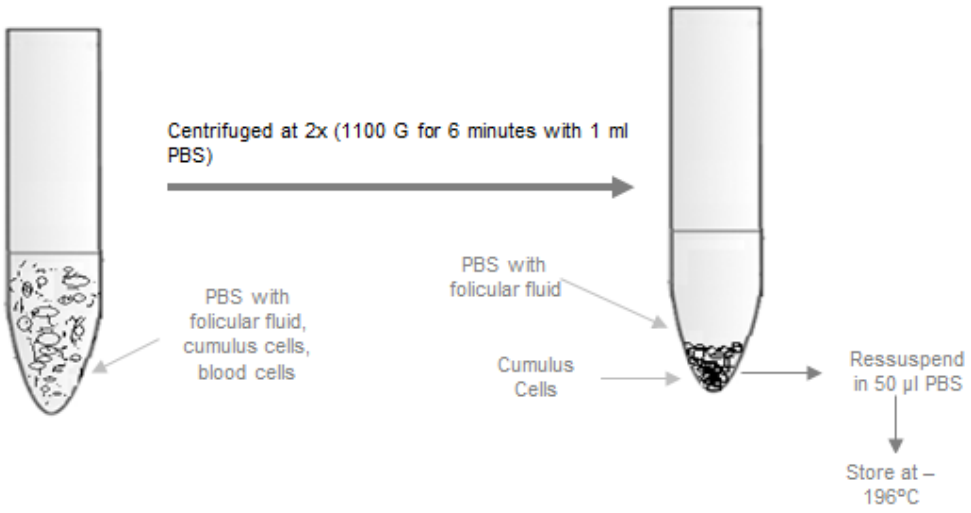

Figure 7- CCs treatment and storage.

2.6.2. BCA CUMULUS CELLS

Extracts were mass normalized using BCA® assay (Fisher Scientific, Loures, Portugal). Briefly, CCs samples were diluted with 50µl SDS 1% and sonicated 3 times for 15 seconds at 60 cycles. In a microplate six controls (increasing concentrations: 0, 1, 2, 5, 10, 20 µg) of BSA were prepared. Then in each microplate well 22.5µl of SDS 1% and 2.5µl of the sample were pipetted, after a quick vortex passage. Finally, all wells were full filled with 200µl of BCA reagents (50:1, Reagent A:B). The microplate was then incubated for 30 minutes at 37ºC before being read at 562nm at the Microplate Reader Infinite M200 (Tecan, Barcelona, Spain). All the reagents used were purchased from Sigma–Aldrich, Portugal.

2.6.3. SAMPLES PREPARATION FOR SDS-PAGE

For Western blot, extracts of 50µg protein were resuspended in 1% SDS and loading buffer (LB). Then were loaded in the gel.SDS-PAGE (14%)

2.6.4. SDS-PAGE (14%)

In SDS polyacrylamide gel electrophoresis (SDS-PAGE) separations were carried out using well established methods (Sambrook, 1989). The percentage and size of the gel used depended on the molecular weight of the proteins that were being separated in the gel.

Table 3| Composition of the running and stacking gels for SDS-PAGE (mini gels).

Components	Running gel (14%)	Stacking gel (3.5%)
Water	5.6 ml	3.3 ml
30% Acryl/8% Bisacryl	9.3 ml	0.6 ml
4x LGB	5.0 ml	---
5x UGB	---	1.0 ml
10% SDS	---	50 µl
10% APS	100 µl	50 µl
TEMED	10 µl	5 µl

A 14% running gel was prepared by sequentially adding the components (Appendix 5) indicated on Table 3 (APS and TEMED were added last, as they initiate the polymerizing process). The solution was pipetted down the spacer into the gel apparatus (Bio-Rad, Portugal), leaving some space for the stacking gel. Then, the water was carefully added to cover the top of the gel and the gel was allowed to polymerize for 1 hour. The stacking gel was prepared according to Table 3. The water was poured out and the stacking gel was added to the apparatus; a comb was inserted and the gel was allowed to polymerize for 30 minutes. After the gel polymerization, the combs were removed, and the wells filled and washed with running buffer. The samples were carefully applied into the wells. The gel was run for 1 hour at 100 V until bromophenol blue from the LB reached the bottom. All the WB reagents were purchased from Sigma–Aldrich, Portugal, except the TEMED (Bio-Rad, Portugal).

2.6.5. IMMUNOBLOTING

For electroblotting, the transfer system tank was used as follows: blotter paper was cut to fit the transfer cassette and a nitrocellulose membrane to fit the gel size. The gel was removed from the electrophoresis apparatus and the stacking gel removed and discarded. The transfer sandwich was assembled immersed in the transfer buffer to avoid trapping air bubbles. The cassette was placed in the transfer apparatus and filled with transfer buffer. Transfer was allowed to proceed for 2 hour at 200 mA. Afterwards, the transfer cassettes were disassembled; the membrane carefully removed and allowed to air dry prior to further manipulations.

In order to visualize the proteins, the blots were probed with a polyclonal anti-cleaved caspase-3 (Asp175) rabbit monoclonal antibody (Cell Signaling Technology, Portugal), which recognizes the

endogenous levels of the large fragment of activated caspase 3 resulting from cleaved adjacent to Asp175. The membranes were soaked in 1xTBS for 5 minutes. Non-specific binding sites were blocked by immersing the membrane in 5% low fat milk in 1xTBST for 1 hour. The membrane was incubated with the primary antibody (anti-cleaved caspase-3; 1:1000), diluted in 3% low fat milk in 1xTBST overnight with shaking at 4ºC. After three washes of 10 min each in 1xTBST, the membrane was incubated with Infrared IRDye-labeled anti-rabbit secondary antibody (LI-COR Biosciences, U.S) (1:5000) in 3% low fat milk in 1xTBST for 1 hour with shaking. The immunodetection was performed using Odyssey Infrared Imaging System.

Control for protein loading was confirmed by staining the membranes with Ponceau S (an example is presented in Figure 15 in Appendix 6) (Romero-Calvo *et al*., 2010).

3. RESULTS

3.1. PARTICIPANTS AND SOCIAL HABITS

The study included 47 couples that underwent IVF treatments in CHVNG, between September 2012 and March 2013. The mean age of women was 32.9 ±3.5 years and of men was 34.4±4.6 years. The mean duration of infertility was 4.1±2.4 years.

Social habits (alcohol, cigarettes and drugs consumption, as well as, physical activity) of participants are summarized in Table 4.

Table 4| Social habits of the participants. (*For statistical analysis this variable was grouped in bigger groups. In alcohol consumption, the social was included in yes group. In physical activity the classes insufficiently active, active and very active were grouped in active.)

Code		Age	Infertility	Years of Inferlity	Smoking habits	Cigarettes/ day	Alcohol consumption*	Drugs consumption	Chemicals/ raditation exposure	Physical Activity*
4556	♂	37	Primary	2	Yes	0 - 5	Socialy	No	No	Very Active
	♀	35	Primary	2	No	0	No	No	No	Active
4839	♂	32	Primary	1	No	0	Socialy	No	No	Inactive
	♀	32	Primary	1	No	0	No	No	No	Inactive
4840	♂	35	Primary	4	Yes	15 - 20	Socialy	No	No	Active
	♀	34	Primary	4	No	0	No	No	No	Active
4841	♂	32	Primary	3	No	0	No	No	No	Active
	♀	31	Primary	3	No	0	No	No	No	Active
4642	♂	39	Primary	4	Yes	0 - 5	No	No	No	Inactive
	♀	37	Primary	4	No	0	No	No	No	Insuficiently Active
4643	♂	29	Primary	7	Yes	05 - 10	No	No	No	Insuficiently Active
	♀	30	Primary	7	Yes	05 - 10	No	No	No	Inactive
4644	♂	39	Primary	7	Yes	05 - 10	Socialy	No	Yes	Inactive
	♀	28	Primary	7	Yes	10 - 15	No	No	No	Inactive
4645	♂	34	Primary	8	Yes	0	Socialy	No	No	Inactive
	♀	33	Primary	8	Yes	05 - 10	Socialy	No	No	Insuficiently Active
4647	♂	35	Primary	11	Yes	0 - 5	Socialy	No	Yes	Inactive
	♀	37	Primary	11	No	0	No	No	Yes	Inactive
4648	♂	39	Primary	5	No	0	Socialy	No	No	Very Active
	♀	37	Primary	5	No	0	No	No	No	Inactive
4649	♂	35	Secondary	2	Yes	05 - 10	Socialy	No	No	Insuficiently Active
	♀	32	Secondary	2	No	0	Socialy	No	No	Inactive

Code		Age	Infertility	Years of Inferlity	Smoking habits	Cigarettes/ day	Alcohol consumption*	Drugs consumption	Chemicals/ raditation exposure	Physical Activity*
4650	♂	37	Primary	6	No	0	No	No	No	Inactive
	♀	34	Primary	6	No	0	No	No	No	Inactive
4651	♂	36	Secondary	2	No	0	Socialy	No	No	Inactive
	♀	34	Secondary	2	No	0	Socialy	No	No	Inactive
4653	♂	40	Primary	4	No	0	No	No	No	Active
	♀	39	Primary	4	No	0	No	No	No	Inactive
4656	♂	28	Primary	5	No	0	No	No	No	Inactive
	♀	27	Primary	5	No	0	No	No	No	Inactive
4658	♂	42	Secondary	1	No	0	Socialy	No	No	Inactive
	♀	34	Secondary	1	No	0	No	No	No	Active
4659	♂	30	Primary	4	No	0	No	No	No	Very Active
	♀	31	Primary	4	No	0	No	No	No	Very Active
4660	♂	34	Primary	4	No	0	Socialy	No	Yes	Inactive
	♀	34	Primary	4	No	0	No	No	No	Inactive
4661	♂	42	Secondary	1	No	0	No	No	Yes	Active
	♀	39	Secondary	1	No	0	No	No	No	Active
4662	♂	39	Primary	3	No	0	No	No	No	Inactive
	♀	35	Primary	3	No	0	No	No	No	Inactive
4663	♂	27	Secondary	4	Yes	10 - 15	No	No	No	Inactive
	♀	33	Secondary	4	No	0	No	No	No	Inactive
4664	♂	40	Primary	3	Yes	15 - 20	No	No	No	Inactive
	♀	24	Primary	3	Yes	15 - 20	No	No	No	Inactive
4667	♂	33	Primary	5	Yes	10 - 15	Socialy	No	No	Inactive
	♀	29	Primary	5	Yes	05 - 10	Socialy	No	Yes	Inactive
4668	♂	42	Primary	1	No	0	No	No	No	Active
	♀	31	Primary	1	No	>20	No	No	No	Active

Code		Age	Infertility	Years of Inferlity	Smoking habits	Cigarettes/ day	Alcohol consumption*	Drugs consumption	Chemicals/ raditation exposure	Physical Activity*
4669	♂	29	Primary	4	No	0	Socialy	No	No	Active
	♀	27	Primary	4	No	0	Socialy	No	No	Active
4670	♂	29	Primary	4	No	0	No	No	No	Insuficiently Active
	♀	29	Primary	4	No	0	No	No	No	Inactive
4671	♂	36	Secondary	3	No	0	Socialy	No	No	Very Active
	♀	38	Secondary	3	Yes	05 - 10	Socialy	No	No	Inactive
4672	♂	37	Primary	3	Yes	15 - 20	Socialy	No	No	Active
	♀	35	Primary	3	Yes	15 - 20	No	No	Yes	Inactive
4673	♂	31	Primary	3	Yes	15 - 20	Socialy	No	No	Insuficiently Active
	♀	32	Primary	3	No	0	Socialy	No	No	Active
4674	♂	33	Primary	4	Yes	0 - 5	Socialy	No	No	Inactive
	♀	31	Primary	4	No	0	Socialy	No	No	Inactive
4675	♂	40	Primary	4	No	0	Socialy	No	No	Inactive
	♀	33	Primary	4	No	0	No	No	No	Inactive
4826	♂	42	Primary	8	Yes	05 - 10	Socialy	No	No	Inactive
	♀	38	Primary	5	Yes	05 - 10	Socialy	No	No	Inactive
4827	♂	41	Primary	3	Yes	15 - 20	Yes	Yes (canabis)	No	Active
	♀	35	Primary	3	Yes	0 - 5	No	No	No	Active
4828	♂	35	Secondary	4	Yes	15 - 20	Socialy	No	No	Very Active
	♀	35	Secondary	4	Yes	0 - 5	Socialy	No	No	Active
4829	♂	32	Secondary	11	No	0	No	No	Yes	Insuficiently Active
	♀	32	Secondary	11	No	0	No	No	No	Active
4830	♂	29	Primary	3	Yes	0 - 5	Socialy	Yes	Yes	Active
	♀	33	Primary	3	No	0	No	No	No	Inactive

Code		Age	Infertility	Years of Inferlity	Smoking habits	Cigarettes/ day	Alcohol consumption*	Drugs consumption	Chemicals/ raditation exposure	Physical Activity*
4831	♂	37	Primary	3,5	Yes	0 - 5	Yes	No	No	Active
	♀	35	Primary	3,5	No	0	No	No	No	Insuficiently Active
4832	♂	28	Primary	2	No	0	No	No	No	Active
	♀	25	Primary	2	No	0	No	No	No	Inactive
4833	♂	29	Secondary	4	Yes	15 - 20	Socialy	Yes	No	Active
	♀	34	Secondary	4	No	0	No	No	No	Insuficiently Active
4834	♂	25	Primary	2	Yes	0 - 5	No	No	Yes	Inactive
	♀	34	Primary	2	No	0	No	No	No	Inactive
4835	♂	31	Primary	3	Yes	0 - 5	Socialy	Yes	Yes	Inactive
	♀	30	Primary	3	No	0	No	No	No	Inactive
4836	♂	35	Primary	5	Yes	15 - 20	No	No	Yes	Very Active
	♀	34	Primary	5	No	0	No	No	No	Active
4837	♂	29	Secondary	1	No	0	Socialy	No	Yes	Active
	♀	32	Secondary	1	No	0	No	No	No	Active
4838	♂	30	Primary	3	No	0	Yes	No	Yes	Active
	♀	30	Primary	3	No	0	No	No	No	Active
4839	♂	31	Primary	9	Yes	5 - 10	Yes	No	Yes	Inactive
	♀	34	Primary	9	No	0	No	No	No	Inactive
4840	♂	38	Primary	5	Yes	0 - 5	Socialy	No	No	Insuficiently Active
	♀	40	Primary	5	No	0	Socialy	No	No	Insuficiently Active
4841	♂	34	Primary	7	Yes	0 - 5	Yes	No	No	Inactive
	♀	32	Primary	7	Yes	5 - 10	Yes	No	No	Inactive

3.2. CLINICAL DATA

In the couples studied 10 have a previous pregnancy (secondary infertility), the others had a primary infertility. The infertility cause was only masculine for 19 cases, only feminine for 10 cases, masculine and feminine for 12 cases and idiopathic for 6 cases. A GnRH antagonist was used in 25 of the cases and for the others was used a GnRH agonist (Table 5). Mean dose of recombinant FSH used was 1830.6±866.8 IU/ml and the mean duration of stimulation cycles was 12.2±2.0.

The cycles were performed with ejaculated sperm and the mean spermatozoa concentration was 33.4±27.7 million/ml. All cycles had positive pick-up with a mean number of 9.9±5.1 oocytes collected. The mean number of inseminated oocytes was 7.9±4.3 and the fertilization rate was 54.9%. Fertilization failed to occur in 7 cases, 5 ICSI cases and in 2 IVF cases.

From the cycles included in the study the embryo transfers was possible in 39 cases (21 ICSI cases and 18 IVF cases), with a mean number of 1.3±0.7 embryos per transfer. A total of 20 pregnancies were achieved (11 ICSI cases and in 9 IVF cases), which corresponded to a clinical pregnancy rate per transfer of 51.3%.The implantation rate was 37.3%.

In the pregnancies from these cycles, 12 births had already happened with a healthy single baby. From remained pregnancies 5 are still ongoing and 3 ended in a spontaneous abortion.

Table 5| Clinical and molecular data.

Code	Technic	Infertility Cause	Stimulation Protocol	Sperm Volume	Sperm Motility	Sperm Morphology	Sperm []spzx10^6	Inseminated Oocytes	Fertilization	Clinical Pregnancy	Ratio Caspase-3
4639	ICSI	Masculine	Antagonist	3,4	70	0	1	6	Yes	Yes	-
4640	ICSI	Masculine	Agonist	3	-	-	0,01	5	Yes	Yes	1,171408
4641	FIV	Idiophatic	Antagonist	3,5	80	13	87	7	Yes	Yes	1,404969
4642	FIV	Masc+Fem	Agonist	2	90	12	52	7	No	No	1,833418
4643	ICSI	Masculine	Agonist	1,2	40	8	56	9	Yes	No	1,887387
4644	ICSI	Masculine	Antagonist	3	80	10	10	9	Yes	Yes	-
4645	FIV	Masc+Fem	Antagonist	3,8	80	8	26	6	Yes	Yes	-
4647	ICSI	Masc+Fem	Antagonist	3,2	60	3	21	3	Yes	No	-
4648	ICSI	Masc+Fem	Agonist	2	60	2	1,5	8	Yes	No	-
4649	FIV	Idiophatic	Antagonist	3	70	12	42	5	Yes	Yes	-
4650	ICSI	Masc+Fem	Agonist	4,5	80	11	61	4	Yes	Yes	-
4651	FIV	Feminine	Agonist	4,8	90	15	58	15	Yes	Yes	1,357336
4653	ICSI	Masc+Fem	Antagonist	2,7	50	25	8	1	No	No	-
4656	FIV	Feminine	Agonist	2,7	90	19	67	14	Yes	No	-
4658	FIV	Feminine	Agonist	2,3	60	10	93	12	Yes	No	-
4659	ICSI	Masculine	Antagonist	1,2	70	8	12	19	Yes	Yes	1,360381
4660	ICSI	Masculine	Antagonist	1,5	50	11	53	3	Yes	No	-
4661	FIV	Feminine	Antagonist	3	70	30	19	12	Yes	Yes	1,196911
4662	ICSI	Masculine	Agonist	0,6	10	0	2,6	4	No	No	-
4663	FIV	Masc+Fem	Agonist	2,5	80	4	70	5	Yes	Yes	1,491239
4664	ICSI	Masculine	Agonist	4	5	0	3,6	6	No	No	-
4667	FIV	Feminine	Agonist	2	70	18	42	10	Yes	Yes	-
4668	ICSI	Masculine	Agonist	3	0	0	6,2	7	No	No	-
4669	ICSI	Masculine	Antagonist	3,4	70	5	45	9	Yes	No	1,690465

Code	Technic	Infertility Cause	Stimulation Protocol	Sperm Volume	Sperm Motility	Sperm Morphology	Sperm []spzx10^6	Inseminated Oocytes	Fertilization	Clinical Pregnancy	Ratio Caspase-3
4670	ICSI	Masculine	Antagonist	2	40	2	25	1	Yes	No	-
4671	FIV	Feminine	Antagonist	1,8	80	24	70	8	Yes	Yes	1,351323
4672	ICSI	Masculine	Antagonist	3,8	60	4	18	14	Yes	Yes	1,127031
4673	ICSI	Feminine	Antagonist	2,3	50	4	15	5	Yes	No	1,432692
4674	FIV	Masc+Fem	Agonist	8	60	16	61	4	Yes	Yes	-
4675	FIV	Feminine	Antagonist	2	60	11	44	12	Yes	No	1,601181
4826	ICSI	Masc+Fem	Agonist	4	50	0	6	9	Yes	Yes	1,420980
4827	ICSI	Masculine	Antagonist	3	20	3	0,4	8	Yes	No	1,597020
4828	ICSI	Masculine	Agonist	3,8	80	10	18	7	Yes	Yes	1,384774
4829	ICSI	Masculine	Agonist	3,8	40	3	0,8	7	Yes	Yes	1,567220
4830	ICSI	Masculine	Antagonist	4	60	7	15	12	Yes	Yes	1,121333
4831	FIV	Masculine	Antagonist	3	80	20	50	17	Yes	No	1,389600
4832	FIV	Feminine	Agonist	4,3	80	10	75	3	No	No	1,332646
4833	FIV	Idiophatic	Agonist	3,5	80	7	30	13	Yes	No	1,563470
4834	ICSI	Masculine	Antagonist	3	10	2	0,04	5	No	No	1,692350
4835	FIV	Idiophatic	Antagonist	2	80	11	17	3	Yes	No	1,601810
4836	FIV	Feminine	Agonist	1,5	50	13	83	7	Yes	No	1,345502
4837	ICSI	Idiophatic	Antagonist	3,5	80	10	51	16	Yes	Yes	1,410690
4838	ICSI	Masc+Fem	Antagonist	3	50	6	48	7	Yes	No	1,479960
4839	ICSI	Masculine	Antagonist	1,5	60	5	0,028	12	Yes	No	1,498019
4840	ICSI	Masc+Fem	Agonist	3,5	75	7	42	8	Yes	No	1,436525
4841	FIV	Idiophatic	Agonist	2	70	14	7	5	Yes	No	1,221543
4556	FIV	Masc+Fem	Antagonist	6	70	7	51	1	Yes	No	1,628478

3.3. CORRELATIONS BETWEEN SOCIAL AND CLINICAL PARAMETERS

All clinical data was correlated with the parameters of lifestyle analyzed and statistical significant correlations had been found.

In the analysis of age parameter no statistical correlation had been found, even between, female or male age with clinical pregnancy ($p>0.05$ and Figure 8).

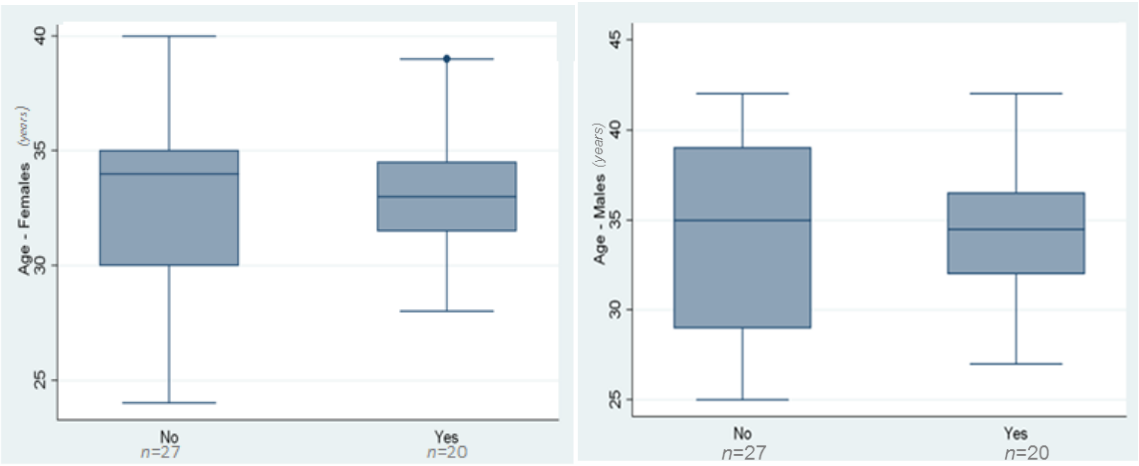

Figure 8- Clinical pregnancy grouped by age (years); female age (years) at left and male age (years) at right.

In the type of infertility a correlation with pregnancy rate had been found and the couples with secondary fertility have better prognosis in achieving clinical pregnancy than the couples with primary infertility ($p\leq0.01$ and Figure 9).

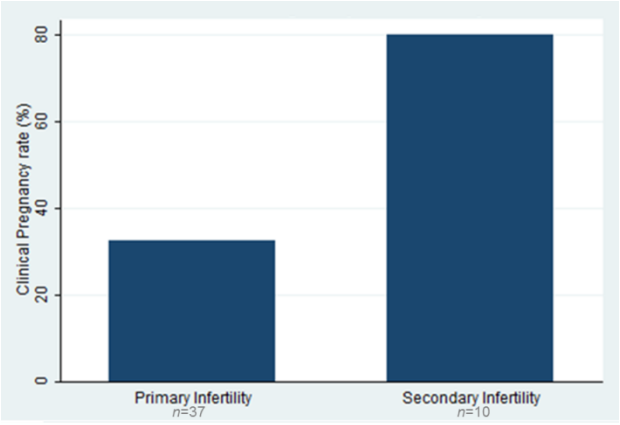

Figure 9- Clinical pregnancy by type of infertility (primary and secondary).

In the women group 1 admitted a daily alcohol consumption and 10 only a social consumption, this two groups were grouped in only one group of consumers to statistical analysis. Alcohol consumption appears to improve fertilization (Figure 10), even though only a statistical tendency is observed (p=0.0931). Otherwise, regarding clinical pregnancy (Figure 10) a borderline statistical significant difference was noted, indicating that pregnancy occurred more frequently in the alcohol consumers group (p=0.050).

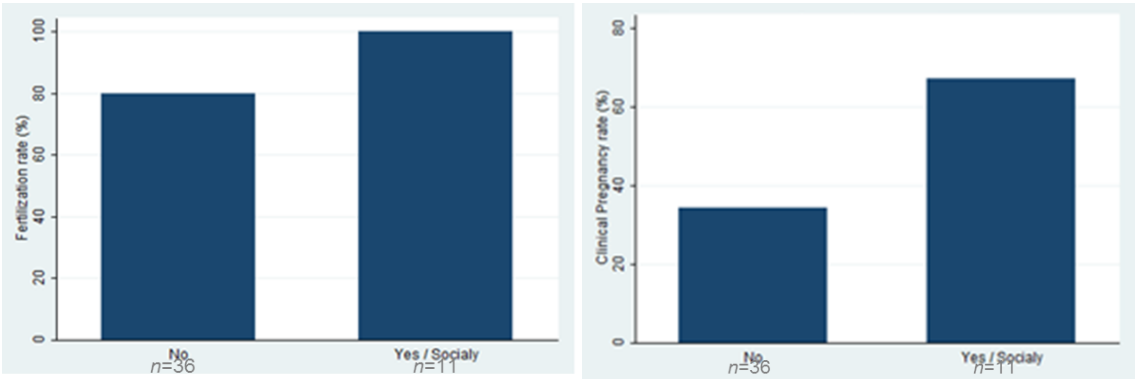

Figure 10- Fertilization rate by alcohol consumption in females, at left. Clinical pregnancy by alcohol consumption in females, at right.

In the men group 5 admitted daily alcohol consumption, while, 25 only refer a social consumes, these two groups were also grouped to statistical analysis. In men, an improvement in the fertilization rate (Figure 11) was observed with alcohol consumption ($p \leq 0.001$). In clinical pregnancy no statistically significant correlation was found.

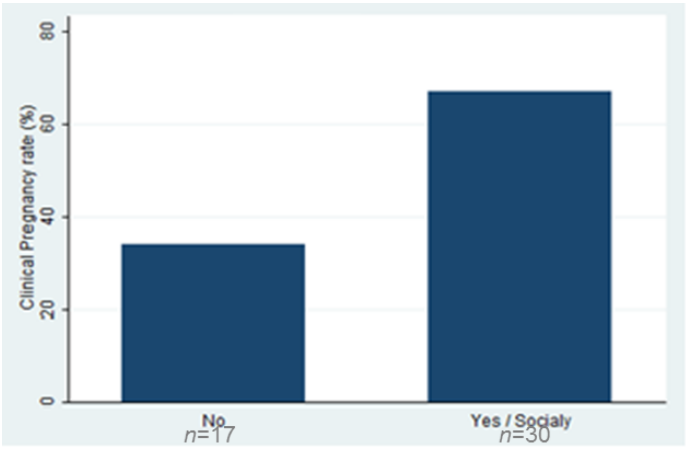

Figure 11- Fertilization rate grouped by alcohol consumption in males.

Smoking habits were present in 12 women and the great majority smoke between 5 to 10 cigarettes per day. In men 25 referred smoking habits, which were fairly distributed between classes of

cigarettes per day. In the statiscal analysis no effects were noted in clinical parameters with smoking habits.

In drugs consumption no women assumed an actual or previously consumption. In men only 4 referred a use of marijuana in during youth, with no actual consume. Drugs consumption also did not show a statistical significant effect on clinical parameters. However, a tendency exists for drugs consumption and spermatozoa concentration (Figure 12), but the number of subjects in consumer group was low to effective conclusions (*n*=4).

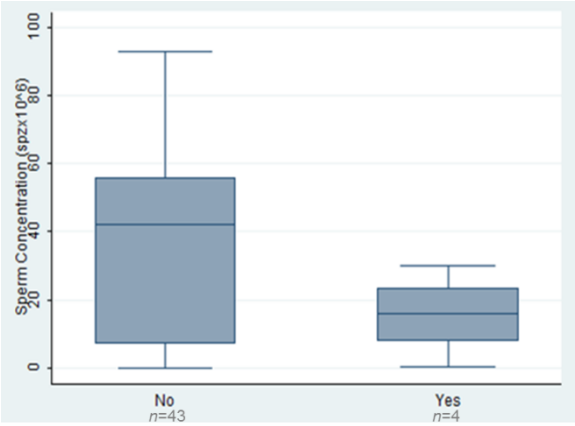

Figure 12- Spermatozoa concentration grouped by drugs consumption in Males.

In the chemicals/ radiation exposure in work place no one referred a ration exposure. However, 3 women and 12 men declared chemicals exposure, in the chemicals mentioned were vinyl chloride, lead, diluents and paints. In this parameter analysis no statistical correlation were found with clinical parameters.

Concerning physical activity, 21 men were inactive, 6 insufficiently active, 12 active and 6 very active. In the groups of activity most of the men were in the inactive group been the other frequencies low. In the correlations of this parameter with clinical parameters no tendency were found.

In women 27 were inactive, 5 insufficiently active, 14 active and only 1 very active, the last three were grouped in active group for statistical analysis. A tendency (p=0.1171) existed for the number of inseminated oocytes increases with physical activity practice (Figure 13). However, this difference was not statistical significant.

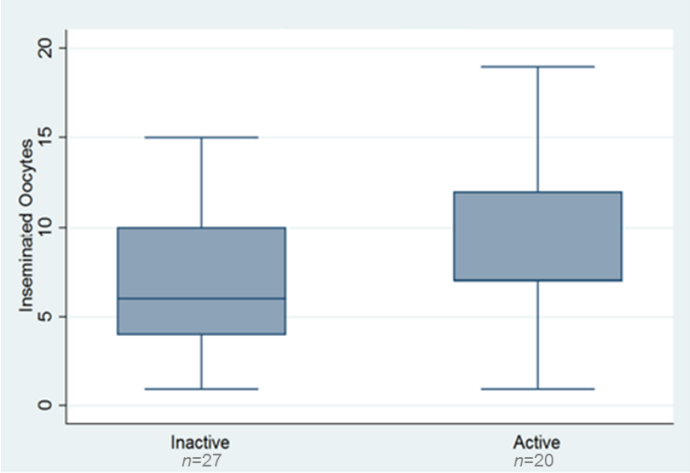

Figure 13- Number of inseminated oocytes grouped by females physical activity.

3.4. MOLECULAR DATA

Cumulus cells integrity was assessed by determining cleaved caspase-3 expression, an apoptotic marker (*n*=30). The typical Western blot signal results of this study are summarized in Figure 14. Differences are evident with respect to the amount of cleaved caspase-3 expressed in CC of pregnant women compared with non-pregnant ones. The mean of cleaved caspase-3 in pregnant group was 1.3 instead 1.5 of the registered in non-pregnant group. Significant ($p<0.01$) higher amount of cleaved caspase-3 was observed in CC of couples who did not achieve (Figure 15). The background of each lane was subtracted to the cleaved caspase-3 levels for statistical analysis purposes. The results suggest an association between achievements of pregnancy and decreased cleaved caspase-3 levels in CC.

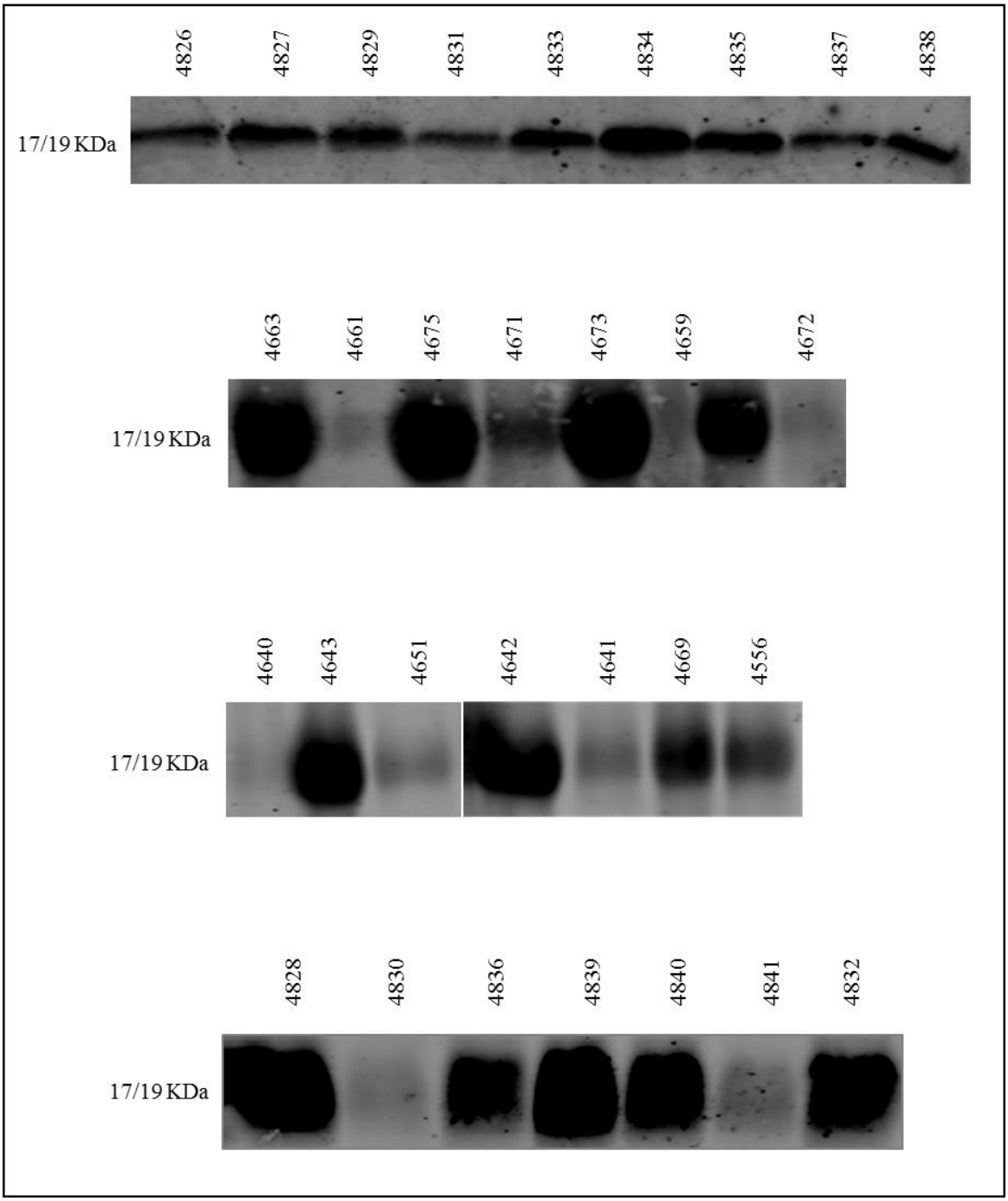

Figure 14- Cleaved Caspase-3 in cumulus cells samples. Cumulus cells extracts (~50µg) were immunoblotted with anti-cleaved caspase-3 antibody (*n*=30).

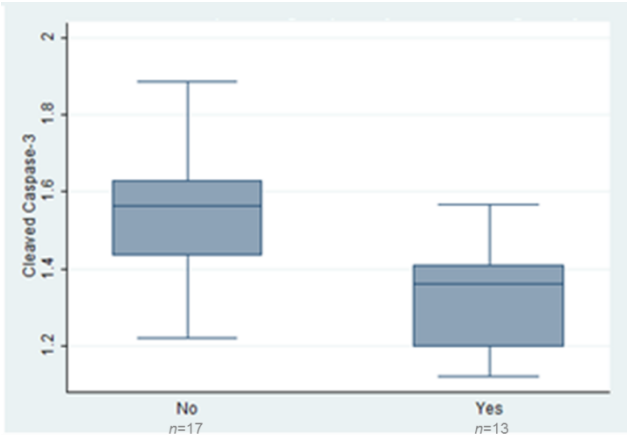

Figure 15- Cleaved Caspase-3 grouped by clinical pregnancy.

There appears to be no tendency between cleaved caspase-3 and women age (Figure 16). Concerning the number of inseminated oocytes, there appears to be an inverse tendency (Figure 16) that is not confirmed by the results of the Spearman Rank Correlation test (Correlation Coefficient: -0.1927; p >0.3076).

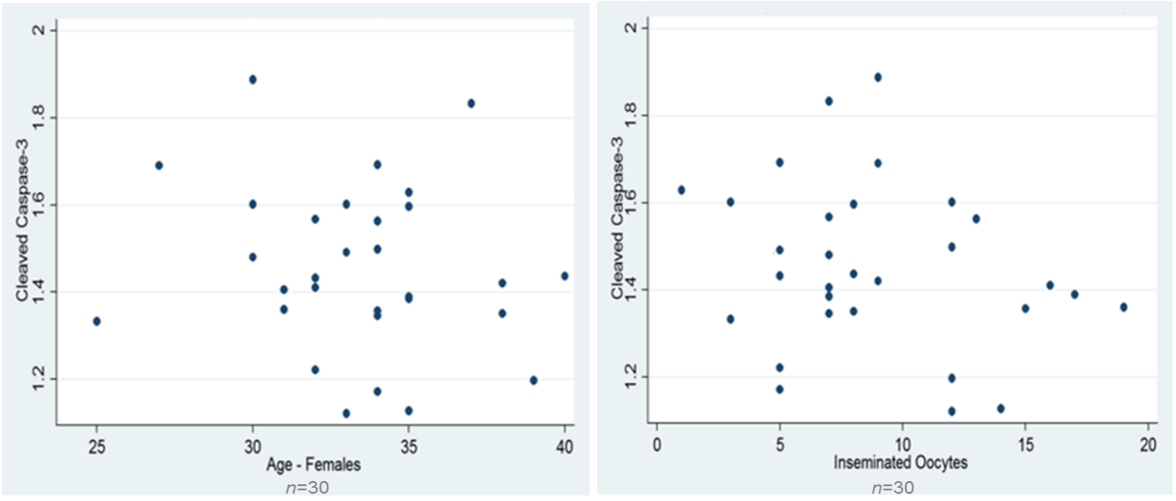

Figure 16- Cleaved Caspase-3 distribution by age at left and by inseminated oocytes at right.

On a brief analysis of some particular cases, samples 4661, 4640, 4672, and 4830 (Table 5 and Figure 14), revealed very low levels of cleaved caspase-3 and achieved pregnancies. Currently 1 pregnancy is ongoing without cause for concerns, in 2 cases the babies have already been born and in 1 the pregnancy results in an abortion. In samples with higher values of cleaved caspase-3 several negative outcomes occurred: a failure of fertilization in IVF occurred in 4642; the IVF resulted in fertilization but the embryos were unable to cleave in 4675; the fertilization and embryo cleavage occurred but resulted in embryos that do not generate a pregnancy in 4643, 4669 and

4556. In some of the higher levels registered in the pregnant group, cases such as 4641 and 4663, the pregnancy ended in abortion.

4. DISCUSSION

4.1. SOCIAL HABITS AND IVF OUTCOMES

It is well know that women suffer an exhaustion of the ovarian follicular pool which results in the end of ovulation process, menopause (La Marca *et al*., 2013). However the women′s fertility potential begins dropping earlier due to oocyte quality deterioration, which is explained, mostly, by the changes in spindle integrity that leads to aneuploidy (ESHRE, 2005). Wang *et al*., 2011, in a review study, concluded that the risk of spontaneous abortion increases to 75% among women over 40 years instead of the 12% registered among women of 20 years. The chromosome abnormalities were responsible for almost 50% of the first trimester miscarriages, being the aneuploidy the most frequent chromosomal abnormality. The human oocyte is highly subjected to aneuploidy due to chromosome missegregation, especially at meiosis I, and the incidence of aneuploidy was correlated with maternal age (Wang *et al*., 2011). However, contrarily to these studies in the present study no negative was found between pregnancy outcomes and higher female ages. Considering that the number of study population was low the results for this parameter cannot be validated.

The men age was also correlated in previous studies, with a decline of fertility. Brahem *et al*., 2011, in a study that included 140 infertile patients (24–76 years) and 50 men with proven fertility (25–65 years) found that increased age of infertile men was associated with a decrease in semen volume, sperm vitality, number of motile spermatozoa and spermatozoa concentration. Sloter *et al*., 2006, performed a study in a population of 97 healthy male (22-80 years) and found that mostly of the man may become progressively less fertile as he ages due to reduced proportions of motile spermatozoa and decreased abilities of motile spermatozoa to maintain forward motion along a linear path. Molina *et al*., 2010, in a retrospective study of 9168 cases (20-77 years) detected a significant decrease in seminal volume, spermatozoa count, motility, viability and normal morphology, and a reduction in alpha-glycosidase and fructose levels in relation to age. Schmid *et al*., 2007, related spermatozoa DNA damage with age increase, which may increase the risks for an unsuccessful and abnormal pregnancy. In all these studies a significant reduction of semen parameters quality and a higher DNA damage was observed, predicting a lower fertility potential. Once again, the low population number was a limitation for this parameter analysis. The study failed to give a significant tendency for ages impact in seminal parameters and pregnancy outcomes.

The results in the present study show a higher probability for couples with secondary infertility to achieve a pregnancy. This makes sense since a pregnancy previous implantation had occurred,

theoretically, should be expected a higher probability of a new implantation. However, no comparative bibliography has been found.

Regarding, alcohol consumption effects in women fertility Jensen *et al*., 1998, performed a study which included 430 couples trying to conceive for the first time and concluded that alcohol intake in women was associated with reduced fecundability. This result was proven even among women who drink five or less drinks for week. Also, Grodstein *et al*., 1994, found a correlation between infertility and moderate alcohol consumption (with an increased number of ovulatory factor and endometriosis). In the present study, a higher fail of fertilization was found in women that consume alcohol. However, the pregnancy in alcohol consumer women shows a borderline statically significant improvement. The women included in the study only 1 assume daily alcohol consumption while the other 10 only referred a social consume of alcohol, what is a really subjective concept and low number of individuals.

Jensen *et al*., 1998, found no correlation for alcohol consumption between men and pregnancy outcomes. Although, Gaur *et al*., 2010, analyzed semen samples of 100 alcoholics and 100 strict nonalcoholic and compared between groups, the presence of astheno, oligozo and teratozoospermia. Their results showed a reduction of normozoospermia amongst alcoholics, the most affected parameters were morphology and spermatozoa. Study concluded that results can be due to deleterious effects of alcohol in male reproductive system as alcohol interferes in the mechanisms of hypothalamus-pituitary-gonadal axis leading to inappropriate production and secretion of LH and FSH that causes Sertoli cells deterioration. The reduction in hormone levels (LH, FSH and testosterone) leads to abnormal morphological development and maturation of spermatozoa, especially in heavy alcoholics. Present study found no difference in semen parameters between alcohol consumers and no consumers. However, is registered an improvement in the pregnancy rate with alcohol consumption. Also, here only 5 men admitted a daily alcohol intakes all the other consumers were social consumers and the subjectivity of this concept could cause some bias in the result obtained.

Attending to smoking habits, Gaur *et al*., 2010, analyzed semen samples of 100 cigarette smokers and 100 non-smoker males and compared in the two groups the seminal parameters analyzed for alcohol consumption (asthenozo, oligo and teratozoospermia). A reduction of normozoospermia amongst is noticed in smokers. Among smokers, a reduction of seminal fluid volume and an increase in viscosity with a delayed liquefaction time were reported. Concerning, smoke habits among women Mello *et al*., 2001 in a nonsystematic literature review concluded that smoke had a negative impact in women fertility. Moreover, than reducing fertility, also, compromises the length

of the gestation and infant birthweight by the action of toxic compounds such as nicotine and carbon monoxide. In the present study, smoking habits showed no effects in clinical parameters.

Marijuana is the recreational drug more used worldwide. Its active substance Δ9-tetrahydocannabinol (THC) binds to the same receptors as endogenous cannabinoids. The endocannabinoid system has a role in the reproductive system, via interaction with two specific receptors (cannabinoid receptor subtype 1 (CNR1) and cannabinoid receptor subtype 2 (CNR2)). In the hormonal axis cannabinoids seem to modulate negatively the activity of LHRH-secreting neurons. In the testicles, the Leydig cells express CNR1. When this receptor is activated, it leads to a reduction in testosterone levels with a consequential inhibition of spermatogenesis and alteration of epididymis and seminal vesicles function, which ultimately results in decreased sperm motility. Furthermore the CRN1 activation also impairs the acrossome reaction (Rossato *et al*., 2008). In women, the endocannabinoid system is required for successful implantation and early pregnancy maintenance. Marijuana consumption can lead to increased difficult in achieving a pregnancy and miscarriages (Kasaru *et al*., 2011). In the present study, no women declared marijuana consumption. However, 4 male partners admitted to had used marijuana previously during youth, the number was to low for an effective conclusion, but a reduction in the sperm concentration looks occurred.

In the industrialized world the exposure to factors that may affect reproductive health increase. Heavy physical work, exposure to anaesthetic fases, anti-neoplastic drugs, heavy metals, solvents, and the many chemicals present in food has a proved role in the decrease of the fertility potential. Many of these chemicals act in the reproductive system as endocrine disruptors (EDC), being the thyroid and the homeostasis of sex steroids the principal targets (Caserta *et al*., 2011). Caserta *et al*., 2011, in a review study concluded that there was significant evidence of EDC exposure being related with alterations in the female fertility and fecundity, as well as trans-generational transfers of undesirable, potentially toxic compounds. In 2010, Perin *et al*., in a study including 400 patients in IVF treatments for the first time due to male infertility tried to correlate the particulate matter found in the air with the IVF success. Their results showed evidence that an exposure to increasing levels of particulate matter during the follicular phase of the conception cycle after IVF is associated with an increased risk of clinical early pregnancy loss. An exposure during the follicular phase of the treatment has a negative impact in stimulation and laboratory parameters. However, studies as the one of Vestergaard *et al*., 2012, that addressed the association between perfluorinated chemicals exposure and the time that a couple needs to get pregnant, included 430 couples, and found no clear association between exposure to these chemicals and longer times to achieve the desirable pregnancy. Similar conclusions were achieved in the review studies of Olea *et al*., 2007,

and Sheiner *et al*., 2003, that defend that more studies are needed to prove the negative action of chemicals in reproductive health that take into the account the complexity of chemicals mixtures and the effects of chemical exposure along different periods of life. In the present study, no clear association between environmental exposures and fertility was found. Still, only 3 women and 12 men referred and exposure to chemicals and/or radiation in the workplace, which was a too low population number.

In the physical activity impact in reproductive potential Gudmundsdottir *et al*., 2009, studied 3887 women (age<45 years) and found an increased risk of infertility among the women with highest levels of frequency or intensity of exercise. However, when comparing the women that practice moderate exercise with sedentary lifestyle women, the risk of infertility decreased in the moderate exercise group. High exercise levels are associated with women who suffer from multiple menstrual dysfunctions such as delayed menarche, amenhorrea, oligomenorrhea and anovulation. These dysfunctions could be linked to the percentage of adipose tissue. Adipocytes secrete factors involved in signaling, energy balance, insulin action, inflammation process and reproductive function. Among them is leptin that mediates the interaction of body fat and reproductive function and is vital for menstruation maintenance. Leptin levels abruptly decline with dietary restrictions as in highly trained athletes. Other adipocytes secreted factor is adiponectin that increases with weight reduction. A high level of this factor (as found in highly trained athletes) contributes to the suppression of LH levels and chronic anovulation (Roupas *et al*., 2011). Present study failed to show a determinant role of physical activity in fertility above women.

In the study of exercise action on male fertility, Wise *et al*., 2011, analyzed 4565 fresh semen samples of 2261 men and observed no correlation in semen parameters alterations and regular exercise practice, except for an association of bicycling≥5 hour/week with lower spermatozoa concentration and total motile spermatozoa. The bicycling secondary effects on fertility looks to be greatly due to scrotal higher temperatures and testicular traumas suffer during sport practice (Southorn, 2002). Also, in men no impact was detected in the present study for this parameter in fertility. In the study group only 3 men practiced bicycling what was a really low number to statical analysis.

4.2. CLEAVED CASPASE-3 AND IVF OUTCOMES

Decreased expression of cleaved caspase-3 ratios as observed in this study was strongly correlated with pregnancy, suggesting its important role as a predictor of IVF success. The oocyte competence

and the follicular development are closely related, since the oocyte development is influenced by the follicular hormonal and molecular environment (Li *et al*., 2008).

Other apoptotic markers had shown their potential role as predictors of fertility in previous studies. Lee *et al*., 2001, performed a study in 34 cycles and tested the presence of apoptosis in cumulus cells (collected at oocyte pick-up) isolated from each oocyte independently by an immunohistochemical test. Their results showed that the presence of apoptotic cells was significantly higher in unfertilized oocytes, poor quality embryos and in the non-pregnant group. Apoptotic cell number increased in patients over 40 years old, agreeing with the lower pregnancy rates registered all over the world in this group. In agreement with the previous evidences, the study from Miao *et al*., 2009, showed that fragmentation caused by Ca^{2+} flow and other signs of apoptosis appear because several cytosolic molecules of apoptosis are present and are important to predict a pregnancy. The study found correlation between oocyte aging and induced apoptosis promoted by factors such as environmental conditions, oocyte interactions with cumulus cells and various chemical components. The aged oocytes showed an altered Ca^{2+} flow, turning the Ca^{2+} signal in an apoptosis-inducing signal. Furthermore, Høst *et al*., 2000, performed a study of apoptotic DNA fragmentation using the free 3'OH DNA termini *in situ* with chemically labeled and unlabeled nucleotides from 21 couples undergoing ICSI due to male factor. Their results showed that low incidence of apoptosis in granulosa and cumulus cells results in better outcome for that follicle oocyte. The degree of apoptosis is also higher in cumulus cells from immature oocytes when compared to metaphase II oocytes.

In IVF treatments to obtain multiple oocytes per cycle clinicians can choose two kinds of drugs which can be administered to recruit follicular development: GnRH agonist or antagonist. Filho *et al*., 2008, used the CC belonging to oocytes which originated transferable embryos and analyzed them for the number of apoptotic cells. In the analyzed group, 9 patients (76 CC) used GnRH agonists and 26 patients (162 CC) used GnRH antagonists. Ages between groups were similar (37±1.6 *vs*. 37±0.8 years), as well as hormonal profiles. The FSH doses administered between groups were not significantly different but the number of oocytes obtained was higher in the agonist group. Concerning the fertilization and pregnancy rate, no significant differences were found and similar results were registered on the percentage of apoptotic cells between groups. However, they found a subtle positive correlation between the percentage of apoptotic cells and estradiol concentration on the day of hCG. This study strongly suggests that the percentage of apoptotic cells does not correlate to the type of IVF protocol and opens the door to more studies about the effectiveness of the correlation between apoptosis and estradiol. The estradiol levels were

not analyzed in this study but the type of IVF protocol showed no effect in the cleaved caspase-3 ratio.

Lifestyle factors such as diet can influence the apoptosis of CC, leading to lower oocyte quality as demonstrated by Wu *et al*., 2010, who studied the impact of high-fat diets in reproduction on mice. Their results showed that the increased lipid accumulation leads to the induction of endoplasmic reticulum stress genes pathways, altered mitochondrial membrane potential, and increased of apoptosis of CC and granulosa cells (biomarkers of lipotoxicity). These results can explain the generally reduced fertilization and pregnancy rates observed worldwidely in obese women.

Varras *et al*., 2012, emphasize the determinant action of survival factors as survivin to promote follicle growth and cellular protection from apoptosis. The gonadotropins and estrogens prevent granulosa cells apoptosis and stimulate their mitotic activity, but androgens enhance granulose cells apoptosis, what can explain lower pregnancy rates in pathologies such as polycystic ovary. Also Fujino *et al*., 2008, found that survivin levels are lower in patients with endometriosis than in patients with only male factor and that the survivin levels are higher in the pregnant patients compared with the non-pregnant ones.

Montfoort *et al*., 2008, performed a study that analyzed the genome-wide expression of CC genes as indicators of embryo viability. They tried to identify differences in gene expression between two kinds of embryos, attending to the fact that early cleavage embryos have better pregnancy prognosis. In the CCs from non-early cleavage embryos, 6 pro-apoptotic genes were overexpressed and 2 anti-apoptotic genes were underexpessed when compared with early-cleavage embryos. The action mechanisms of these genes are not clear but this study suggests that the apoptotic signal can easily be transferred from the cumulus genes to the oocyte through the gap junctions.

5. FINAL CONCLUSIONS

The reproductive potential is definitely influenced by the lifestyle and environment. New problems are arising with the literacy and professional careers that lead maternity to latter ages, when fertility is reduced and the risk to chromosomal aberrations is very high.

The phenomena of economic crisis seems to worsen this problem, because with the difficulty to find a job, the insecurity and budget limit leads couples to delay the paternal project. The concerns about the environmental exposures are not new and multiple studies have been performed before. From the multiple advices from scientists, the international laws are becoming stricter on pollution and on use of some compounds that can act as endocrine disruptors. However, every law restrictions and publicity around the collateral effects of alcohol, tobacco and drugs consumption is not yet sufficient to create a social conscience and stop the increasing consume numbers. The use of these substances results in serious damages to our reproductive potential.

The present study failed to prove the effects of those substances on fertility due to the limit number of couples attending the study. However, the levels of cleaved caspase-3 in cumulus appear to be a good marker of oocytes quality and a predictor of fertilization and outcome.

The great correlation between the cleaved caspase-3 levels and pregnancy achievement leads to new questions. The clear understanding of how apoptotic signal emerges and acts may enable new therapeutics to protect oocytes from the harmful follicular ambient.

Further studies are needed to explore if the solution could pass through the finding of new therapeutic targets that minimize the oocytes environment toxicity or through the establishment of an i*n vitro* maturation medium that takes the oocyte away from the molecular signaling in the follicle to a controlled environment.

Concerning to limitations of the study the main methodological limitation was the small sample size, specially, to evaluate parameters with such a wide inter-subject variability. Also, some concepts as social alcohol consumption should be better define to become less subjective.

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APPENDIX 1

SOME STUDIES PUBLISHED IN THE LAST FIVE YEARS ABOUT OOCYTE QUALITY MARKERS

	Article	Study	Conclusions
Biochemistry	Anderson *et al.*, 2009	BNDF GREM1	Relationships between BDNF expression and fertilization were identified, and the potential value of GREM1 expression as a marker of embryo quality.
	Anifandis *et al.*, 2010	25-OH vitamin D Glucose	The data suggests that excess serum and follicular fluid vitamin levels in combination with decreased follicular fluid glucose levels have a detrimental impact on the IVF outcome.
	Altun *et al.*, 2011	Interleukin 6 (IL-6)	Lower follicular fluid IL-6 levels in IVF patients are associated with increased likelihood of clinical pregnancy.
	Babayan *et al.*, 2008	Hyaluronan (HA)	HA in follicular fluid is decreased in women with unsuccessful implantation or with an endocrine disorder. A woman's level of HA production may influence the potential for implantation of her embryos.
	Bedaiwy *et al.*, 2012	FF reactive oxygen species (ROS) levels, total antioxidant capacity (TAC) and ROS-TAC score	Higher FF TAC, higher FF ROS-TAC scores and lower FF ROS levels are associated with pregnancy after ICSI.
	Bódis *et al.*, 2010	L-arginine Methylarginines	Elevated levels of follicular fluid l-arginine and methylarginines appear to have an adverse influence on the reproductive processes as reflected by a reduction in the number of oocytes and embryos conceived.
	Browne *et al.*, 2009	High Density Lipoprotein (HDL)	The study found a negative correlations between multiple FF HDL components and embryo fragmentation.
	Filali *et al.*, 2009	Incidence of apoptosis in CCs	Results suggest that BCL2 expression is strongly associated with the ability of oocytes to complete nuclear maturation and to be fertilized.
	Gode *et al.*, 2011	Growth differentiation factor (GDF)- 9	Higher mature GDF9 levels in the follicular fluid were significantly correlated with oocyte nuclear maturation and embryo quality.
	Jeppesen *et al.*, 2012	LH	LH appears to affect human follicular development during most the follicular phase in normal women.

	Article	Study	Conclusions
	Kralikova *et al.*, 2011	Homocysteine (Hcy)	High follicular fluid Hcy levels are negatively correlated with oocyte fertilization and the quality of embryo, which indicates that follicular fluid Hcy may play an important role in the development of oocytes and fertilization.
	Kedem-Dickman *et al.*, 2012	AMH expression and secretion	AMH is highly expressed in and secreted from cumulus GC of preovulatory follicles containing premature and atretic oocytes.
	Lédée *et al.*, 2008	Interleukin (IL-2) Interferon (IFN-γ) Interleukin (IL-12) Chemokine CCL5 Granulocyte colony-stimulating factor (G-CSF)	Significantly higher levels of interleukin (IL-2) and interferon (IFN-γ) were detected in FF for embryos that underwent early cleavage. IL-12 was significantly higher in FF corresponding to highly fragmented embryos and the chemokine CCL5 was significantly higher in FF related to the best quality (Top) embryos. The level of granulocyte colony-stimulating factor (G-CSF) in individual FF samples was correlated with the implantation potential of the corresponding embryo.
	Maman *et al.*, 2012	LH receptor (LHR) expression in human granulosa cells	Overexpression of LHR in cumulus GCs of MII oocytes may signal malfunction of oocytes and low fertilization capacity.
	Matos *et al.*, 2009	Superoxide Dismutase (SOD)	Successful ART was associated with higher SOD activity.
	Ocal *et al.*, 2012	FF homocysteine levels	Low follicular fluid homocysteine level is associated with a better chance of clinical pregnancy.
	Ouandaogo *et al.*, 2012	Transcriptome profiles of CCs	CC transcriptomic signature varies according to both the oocyte maturation stage and the maturation conditions.
	Pasqualotto *et al.*, 2009	Superoxide dismutase (SOD) Catalase	Fertilization and cleavage rates were correlated with the levels of SOD and Catalase in the follicular fluid (decrease in oocyte fertilization under conditions of reduced antioxidant capacity).
	Richards *et al.*, 2012	Adiponectin	Adiponectin can modulate not only follicle growth but also embryo development in mice and humans.
	Savchev *et al.*, 2010	Vascular endothelial growth factor (VEGF)	Results suggest that elevated VEGF(165) levels are associated with less favorable patient characteristics and clinical IVF outcomes.

	Article	Study	Conclusions
	Tamura *et al.*, 2012	Melatonin (N-acetyl-5-methoxytryptamine)	Melatonin have benneficial effects on reproductive physiology, acting as an anti-oxidant to reduce oxidative stress in oocyte maturation and embryo development.
	Wallace *et al.*, 2012	Metabolomic analysis of FF	Differences in the metabolite composition of follicular fluid correlate with the developmental competence of the human oocyte.
	Zamah *et al.*, 2010	Epidermal Growth Factor (EGF)-like Amphiregulin (AREG)	Gonadotrophin stimulation of the human ovulatory follicle produces massive accumulation of the EGF-like growth factor AREG in FF. So EGF-like growth factors play a role in critical peri-ovulatory events in humans, and AREG accumulation is a useful marker of gonadotrophin stimulation and oocyte competence.
Genetics	Assidi *et al.*, 2011	Genomic biomarkers in CCs	Genomic markers are a powerful reinforcement of morphological approaches of oocyte selection. Their large-scale validation could increase pregnancy outcome and single embryo transfer efficiency.
	Andriaenssens *et al.*, 2011	CC gene expression	Decreased HAS2, ALCAM and PTGS2 seem to coincide with reduced oocyte competence.
	Fragouli *et al.*, 2012	SPSB2 and TP53I3	Study speculates that both these genes might have the potential to serve as non-invasive markers of oocyte aneuploidy.
	Gebhardt *et al.*, 2011	CC gene expression	Cumulus cell VCAN, PTGS2, GREM1, and PFKP expression may identify oocytes with high developmental potential, leading to enhanced implantation rates and greater developmental capacity throughout gestation.
	Lee *et al.*, 2010	CKB PRDX2	Expression of these genes in cumulus cells was associated with embryo quality. CKB and PRDX2 may serve as biomarkers or therapeutic targets for the developmental potential of oocytes.
	Wathlet *et al.*, 2012	Gene expression in CC	EFNB2 and CAMK1D are promising genes that could help to choose the embryo to transfer with the highest chance of a pregnancy.

APPENDIX 2

Authorization from the CNPD

COMISSÃO NACIONAL
DE PROTECÇÃO DE DADOS

Processo n.º 3900/2012

AUTORIZAÇÃO N.º 4850 **/2012**

I. Do Pedido

O **Laboratório da Transdução de Sinais, Centro de Biologia Celular da Universidade de Aveiro** notificou à CNPD um tratamento de dados pessoais com a finalidade de elaborar um estudo observacional para avaliar os marcadores bioquímicos no líquido folicular e nas células de cúmulos do ovócito.

Serão incluídos no estudo os casais e pacientes em ciclo de fertilização *in vitro* que recebam acompanhamento na Unidade de Medicina da Reprodução do Centro Hospitalar de Vila Nova de Gaia/Espinho, EPE.

A participação no estudo consiste na utilização do fluído folicular, células de cúmulos dos ovócitos e sémen excedentário, bem como na recolha de dados do processo clínico e na resposta pelo titular dos dados a um questionário sobre hábitos de vida.

As análises laboratoriais utilizarão o material biológico excedentário, no âmbito das análises requeridas pelos médicos assistentes, antes do mesmo ser destruído, não sendo criado um biobanco.

O Embriologista assistente, investigador no estudo, solicitará consentimento informado, cuja declaração será arquivada no processo clínico dos doentes.

Os dados serão recolhidos num caderno de recolha de dados em formato papel pelo Embriologista assistente.

No "caderno de recolha de dados" não há identificação nominal do titular, sendo aposto um código de doente. A chave desta codificação só pode ser conhecida da equipa de investigação.

Os destinatários serão ainda informados sobre a natureza facultativa da sua participação e garantida confidencialidade no tratamento.

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COMISSÃO NACIONAL
DE PROTECÇÃO DE DADOS

II. Da Análise

A CNPD já se pronunciou na sua Deliberação n.º 227 /2007 sobre o enquadramento legal, os fundamentos de legitimidade, os princípios orientadores para o correto cumprimento da Lei de Protecção de Dados, bem como as condições gerais aplicáveis ao tratamento de dados pessoais para esta finalidade.

No caso em apreço, a notificação enquadra-se no âmbito tipificado por aquela Deliberação.

A informação tratada é recolhida de forma lícita (art.º 5º, n.º1 al. a) da Lei 67/98), para finalidades determinadas, explícitas e legítimas (cf. al. b) do mesmo artigo) e não é excessiva.

O fundamento de legitimidade é o consentimento expresso do titular dos dados.

III. Da Conclusão

Assim, nos termos das disposições conjugadas do n.º 2 do artigo 7.º, n.º1 do artigo 27º, al. a) do n.º 1 do artigo 28º e art. 30º da Lei de Protecção de Dados, com as condições e limites fixados na referida Deliberação n.º 227/2007, que se dão aqui por reproduzidos e que fundamentam esta decisão, e ainda com a condição aqui fixada, autoriza-se o tratamento de dados supra referido, para a elaboração do presente estudo.

Termos do tratamento:
Responsável pelo tratamento: Laboratório da Transdução de Sinais, Centro de Biologia Celular da Universidade de Aveiro
Finalidade: Estudo observacional para avaliar os marcadores bioquímicos no líquido folicular e nas células de cúmulos do ovócito.
Categoria de Dados pessoais tratados: código do participante, dados demográficos (Idade, Sexo, peso, altura e profissão), hábitos tabágicos, consumo de álcool e drogas, prática desportiva, causa de infertilidade, tipo de indução da ovulação, n.º de folículos com diâmetro igual ou superior a 16 mm, n.º de ovócitos colhidos, taxa de fecundação de ovócitos, qualidade embrionária e taxa de gravidez.
Entidades a quem podem ser comunicados: Não há.
Formas de exercício do direito de acesso e retificação: Junto do embriologista assistente.
Interconexões de tratamentos: Não há.

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Transferências de dados para países terceiros: Não há

Prazo de conservação: A chave de codificação dos dados do titular e as amostras biológicas devem ser destruídas um mês após o fim do estudo.

Dos termos e condições fixados na Deliberação n.º 227/ 2007 e na presente Autorização decorrem obrigações que o responsável deve cumprir. Deve, igualmente, dar conhecimento dessas condições a todos os intervenientes no circuito de informação.

Lisboa, 04 de Junho de 2012

Ana Roque (Relatora), Luís Paiva de Andrade, Vasco Almeida, Helena Delgado António, Luís Barroso

Filipa Calvão (Presidente)

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APPENDIX 3

INFORMED CONSENT

CENTRO HOSPITALAR
VILA NOVA DE GAIA|ESPINHO

universidade de aveiro theoria poiesis praxis

Consentimento informado

A Unidade de Medicina da Reprodução Drª Ingeborg Chaves encontra-se a desenvolver um estudo de marcadores bioquímicos no líquido folicular, células de cúmulus do ovócito e esperma em parceria com a Universidade de Aveiro.

O líquido folicular é o meio que envolve o ovócito durante a maturação deste no ovário, sendo que, o objectivo do presente estudo é poder usar o líquido folicular e as células do cumulus para investigação de marcadores bioquímicos antes de este ser descartado. Assim como, no esperma excedentário que é descartado no decurso das técnicas.

A participação neste estudo não acarreta qualquer encargo para o casal e para a instituição, nem altera de forma alguma o decurso natural das técnicas de procriação medicamente assistida. As amostras serão identificadas a partir de códigos numéricos, garantindo total confidencialidade e anonimato dos dados dos intervenientes. Os casais que aceitarem ser incluídos neste estudo terão a liberdade de em qualquer altura remover a sua autorização, sendo automaticamente excluídos.

Para participar no estudo pedimos apenas que responda a algumas questões e assine este consentimento.

_____

_____

A equipa da UMR do CHVNG/ E.P.E Espinho agradece desde já a sua colaboração!

APPENDIX 4

SURVEY FOR PARTICIPANTS IN THE STUDY

CENTRO HOSPITALAR VILA NOVA DE GAIA|ESPINHO

universidade de aveiro theoria poiesis praxis

Código do casal:_____

Inquérito para os intervenientes no estudo

♂	♀
Idade:_____ Tipo de Infertilidade[1]: Primária □ Secundária □ Nº de anos:__	Idade:_____ Tipo de Infertilidade: Primária □ Secundária □ Nº de anos:__
Profissão: _____	Profissão: _____
É fumador? Não □ Sim□ Quantos cigarros costuma fumar por dia? _____	É fumador? Não □ Sim□ Quantos cigarros costuma fumar por dia? _____
É consumidor de álcool? Não □ Sim□.....Socialmente □	É consumidor de álcool? Não □ Sim□.....Socialmente □
É consumidor de drogas? Não □ Sim□.....Qual:_____	É consumidor de drogas? Não □ Sim□.....Qual:_____
No seu local de trabalho encontra-se exposto a algum tipo de químico ou radiação[2]? Não □ Sim□.....Qual:_____	No seu local de trabalho encontra-se exposto a algum tipo de químico ou radiação? Não □ Sim□.....Qual:_____
Pratica exercício físico? Não □ Sim□.....Qual:_____ Frequência semanal:_____	Pratica exercício físico? Não □ Sim□.....Qual:_____ Frequência semanal:_____

[1] Infertilidade primária – Nunca engravidou.
Infertilidade secundária – Já teve uma gravidez.
[2] Alguns exemplos de agentes químicos: pesticidas, chumbo, mercúrio, etc.
Alguns exemplos de radiações: raios- X, radioactividade, etc.
Appendix 5

APPENDIX 5

SOLUTIONS FOR WESTERN BLOT

LGB **(Lower Gel Buffer)**	To 900 ml of deionized H_2O add: Tris 181.65 g SDS 4 g Mix until the solutes have dissolved. Adjust the pH to 8.9 and adjust the volume to 1 L with deionized H_2O.
UGB **(Upper Gel Buffer)**	To 900 ml of deionized H_2O add: Tris 75.7 g Mix until the solute has dissolved. Adjust the pH to 6.8 and adjust the volume to 1 L with deionized H_2O.
30%Acrylamide/ 0.8%Bisacrylamide	To 70 ml of deionized H_2O add: Acrylamide 29.2 g Bisacrylamide 0.8 g Mix until the solutes have dissolved. Adjust the volume to 100 mL with deionized H_2O. Filter through a 0.2 μm filter and store at 4 °C.
10% APS (ammonium persulfate)	In 10 ml of deionized H_2O dissolve 1 g of APS.
10% SDS **(sodium dodecilsulfate)**	In 10 ml of deionized H_2O dissolve 1 g of SDS.
4x Loading gel buffer	1 M Tris solution (pH 6.8) 2.5 ml (250 mM) SDS 0.8 g (0.8%) Glycerol 4 ml (40%)

	β-Mercaptoethanol 2 ml (2%) Bromophenol blue 1 mg (0.01 %) Adjust the volume to 10 ml with deionized H_2O. Store in darkness at RT.
1 M Tris (pH 6.8) solution	To 150 ml of deionized H_2O add: Tris base 30.3 g Adjust the pH to 6.8 and adjust the final volume to 250 ml.
10x Running buffer	Tris 30.3 g (250 mM) Glycine 144.2 g (2.5 M) SDS 10 g (1%) Dissolve in deionized H_2O, adjust the pH to 8.3 and adjust the volume to 1 L.
10x Transfer buffer	Tris 3.03 g (25 mM) Glycine 14.41 g (192 mM) Mix until solutes dissolution. Adjust the pH to 8.3 with HCl and adjust the volume to 800 ml with deionized H_2O. Just prior to use add 200 ml of methanol (20%).
10x TBS (Tris buffered saline)	Tris 12.11 g (10 mM) NaCl 87.66 g (150 mM) Adjust the pH to 8.0 with HCl and adjust the volume to 1 L with deionized H_2O.
10x TBST **(TBS + Tween)**	Tris 12.11 g (10 mM) NaCl 87.66 g (150 mM) Tween 20 5 ml (0.05%) Adjust the pH to 8.0 with HCl and adjust the volume to 1 L with deionized H_2O.

Developer Solution	GBX developer 220 ml Adjust the volume to 1 L with deionized H_2O.
Fixation Solution	GBX fixer 220 ml Adjust the volume to 1 L with deionized H_2O.

APPENDIX 6

CONTROL FOR PROTEIN LOADING BY STAINING THE MEMBRANES WITH PONCEAU S

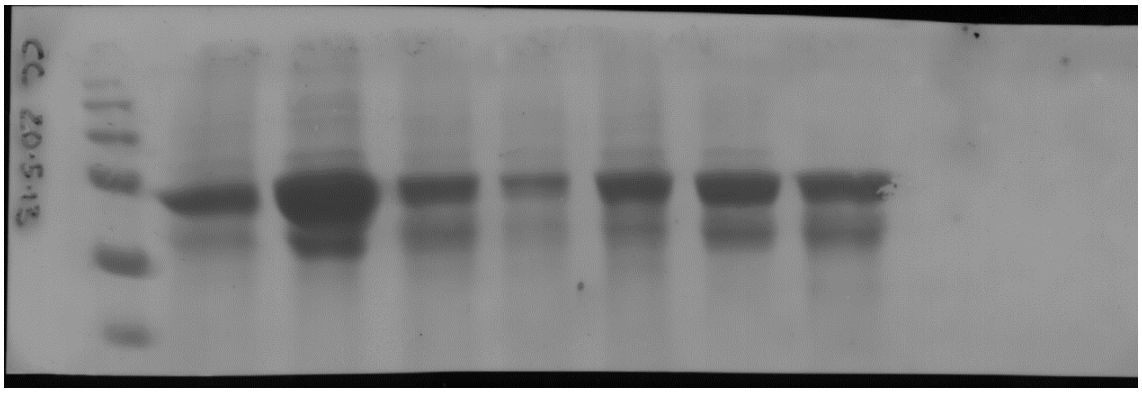

Figure 17- Control for protein loading by staining the membranes with Ponceau S.